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Interactions of *Vaccinia virus* with the host cell

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Declaration

This thesis has been composed by the student, and either the work is the student's own, or, if the student has been a member of a research group, the student has made a substantial contribution to the work, such contribution being clearly indicated, and the work has not been submitted for any other degree or professional qualification except as specified.

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Contents

Abstract

Vaccinia virus (VACV) is a double-strand DNA virus belonging to Orthopoxvirus genus of the family *Poxviridae*. After entry VACV replicates entirely in cytoplasm of the host cell, using virally-encoded DNA replication and transcription machinery. Nevertheless, a recent siRNA screen revealed that the nuclear export protein nucleoporin 62 (NUP62) plays a role in VACV morphogenesis, suggesting VACV requires the host cell nucleus for virus replication. In order to investigate this further we analysed the effect of two chemical inhibitors of nuclear-cytoplasmic transport, leptomycin B and 335, on the replication of VACV in HeLa and A549 cells. No significant difference was detected in viral titre when nuclear export was inhibited with drug treatment, as this resulted in only a three-fold reduction in virus titre. In contrast, both drugs reduced the yield of influenza virus (which is known to be dependent on nuclear-cytoplasmic transport) by 200 fold. These results indicate that VACV is able to replicate to near normal titres in cells with reduced nuclear export. A siRNA interference study also uncovered MAST3 (microtubule-associated serine/threonine kinase 3) as a putative pro-viral cellular protein. MAST3 contains a serine/threonine kinase domain and a PDZ protein interaction domain. The present study demonstrated a statistically significant reduction of VACV replication in HeLa cells in the presence of MAST3-targeted siRNA. This work provides new information on the complex interactions between VACV and the host cell.

Lay summary

Most viruses including influenza virus require the nucleus of their host cell, therefore virus replication can be blocked by inhibiting nuclear-cytoplasmic transport via disruption of the nucleus pore structure. However, one exception is the poxvirus family, a family of DNA viruses whose life cycle occurs entirely in the area called the virus factory in cytoplasm. Thus no drug targeting to nuclear-cytoplasmic transport is supposed to affect poxviruses. However, a recent poxvirus study suggested that virus yield can be reduced by inhibiting virus maturation using the siRNA knockdown technique to remove one element of the nucleus pore complex, nucleoporin 62. In order to investigate this further, we established a nuclear-cytoplasmic trafficking interfering experiment in which HeLa cells and A549 cells were treated with two drugs of nuclear -cytoplasmic transport, Leptomycin B and 335, following infection of Vaccinia virus (VACV), a family member of poxvirus. Drug treatment resulting in only a non-significant three-fold reduction in VACV virus growth, suggesting that VACV is able to replicate to near normal titres in cells with reduced nuclear-cytoplasmic transport. In comparison, in a parallel study influenza virus replication was reduced by 200 fold. In addition to nuclear-cytoplasmic transport, a siRNA interference study also uncovered a cellular protein MAST3 (microtubule-associated serine/threonine kinase 3) that may take part in virus replication. MAST3 is a cellular signaling messenger MAST3 contains a serine/threonine kinase domain for catalytic function and a PDZ protein interaction domain as a binding site. The present study demonstrated a statistically significant reduction of VACV replication in HeLa cells in the presence of MAST3-targeted siRNA. This work provides new information on the complex interactions between VACV and the host cell.

Abbreviations

VARV-Variola virus

VACV- Vaccinia virus

IMV - Intracellular mature virus

MTOC - microtubule organizing centre

TGN - trans-Golgi network

IEV - intracellular enveloped virus

CEV - cell-associated enveloped virus

EEV - extracellular enveloped virus

NF-κB - Nuclear factor kappa B

MAP- microtubule-associated protein

IκBα - inhibitor of kappa B alpha

IL - interleukin

TNF - tumor necrosis factor

TLRs - Toll-like receptors

MyD88 - myeloid differentiation factor 88

TRAF6 - TNF receptor-associated factor 6

IRAK2 - IL-1 receptor-associated kinase 2

IKK - kappa B kinase

siRNA - interfering RNA

NUPs - nucleoporins

NLS - nuclear localization signal

NES - nuclear export signal

CRM1- chromosome region maintenance 1, also known as exportin 1

NXF1 - TIP associated protein TAP

RanGAP1 - GTPase-activating protein1

RCC1 - Regulator of chromosome condensation 1

HRV - Human rhinovirus

PV - polivovirus

EMCV - Encephalomyocarditis virus

ISRE - interferon stimulated response elements

ORF6 - open reading frame 6

SARSCoV - severe acute respiratory syndrome coronavirus

AdVs - Adenoviruses

E4orf6 - open reading frame 6

CMC - carboxymethyl cellulose

1. Introduction

1.1 Poxviridae

Poxviridae is a family of large, double stranded DNA viruses with a broad range of hosts including vertebrates and arthropods. There are two subfamilies, Chordopoxvirinae and Entomopoxvirinae, which including 28 genera and 69 species. The 10 genera in the Chordopoxvirinae subfamily contain species that infect mammals, and include the genera Orthopoxvirus, Parapoxvirus, Avipoxivrus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxivrus genus. Entomopoxvirinae is a subfamily of poxviral species infecting insects, including Alphaentomopoxvrius genus, Betaentomopoxvrius genus and Gammaentomopoxvirus genus (Bernard N Fields; David M Knipe (David Mahan) 2001).

1.2 Vaccinia virus

Vaccinia virus (VACV) belongs to the Orthopoxvirus genus along with Variola virus (the causative agent of smallpox), Monkeypox virus and Ectromelia virus (Bernard N Fields; David M Knipe (David Mahan) 2001). VACV encodes a 190kbp linear genome compacted in the core of the virus (Gubser, Hue et al. 2004). The ellipsoidal shaped virion mature virion has major and minor axes measuring 320 to 380nm and 260 to 340nm respectively (Malkin, McPherson et al. 2003). In the 19th and 20th centuries, VACV was widely used as a vaccine against smallpox, resulting in the complete eradication of this disease. Today studies into VACV continue as it is an excellent model to look at virus-host interactions, for example it interferes the innate immune system of the host through a wide range of novel immunomodulatory virus proteins (Smith, Benfield et al. 2013) and modulates the host cell cytoskeleton (Leite and Way 2015) VACV and attenuated strains such as modified Vaccinia virus Ankara (MVA) are also being developed as vaccine vectors against a variety of human and animal diseases including HIV, malaria, tuberculosis and cancer, and as an oncolytic agents (Gomez, Najera et al. 2011).

1.3 Poxvirus disease and symptoms

Smallpox was the most serious poxvirus disease in humans, caused by Variola virus (VARV). It was spread by inhalational exposure of oral, nasal or pharyngeal droplets that contained the

virus and initially caused influenza like symptoms such as fever of at least 38.3°C, muscle pain, malaise, headache and prostration. After 10-14 days incubation the classic poxvirus rash would appear on the face, all four limbs, mucous membranes of the mouth, tongue, palate and throat. When visible lesions were first observed, the temperature fell to near normal, and the lesions rapidly enlarged and ruptured, developing from macules to papules and eventually pustules that dried up and crusted over by 14 to 16 days if the patient survived. By day 16-20, scabs would form over all the lesions and flake off, leaving depigmented scars (Bernard N Fields; David M Knipe (David Mahan) 2001). Smallpox was declared eradicated in 1980 due to a highly successful worldwide vaccination programme using VACV.

VACV causes a much milder disease than VARV, characterised by rash, fever, headache, and muscle pain. The virus replicates at the site of inoculation, forming local erythematous maculopapules. These maculopapules then vesiculate, scar and heal over by 10-14 days (Bernard N Fields; David M Knipe (David Mahan) 2001). Poxviruses are cross-protective within genus, consequently prior infection with VACV protects against infection with VARV and therefore smallpox. This was the basis of the smallpox vaccination programme.

1.4 Virus life cycle

During the VACV lifecycle four different types of VACV virions are produced within the cytoplasm of the cell (Figure 1.1). Intracellular mature virions (IMV) surrounded by a single membrane are the first and most numerous virion produced. The vast majority of IMVs remain within the cytoplasm in viral factories until cell lysis, however a small percentage of them are transported to sites near the microtubule organizing centre (MTOC) where they obtain a double-layer of membrane that is derived from either the trans-Golgi network (TGN) (Smith and Law 2004) or early endosomes (Tooze, Hollinshead et al. 1993, van Eijl, Hollinshead et al. 2000), forming intracellular enveloped virus (IEV) with a total of three membranes. IEVs then travel to the cell periphery using microtubules (Ward and Moss 2001, Risco, Rodriguez et al. 2002). Once IEV approaches the cell surface the outer membrane of IEV fuses with the plasma membrane, which exposes the enveloped virion on the cell surface; the virion is now known as a cell-associated enveloped virus (CEV) and has a total of two membranes. On the cell surface, CEVs can be propelled towards uninfected cells by actin tails forming beneath the plasma membrane (Payne 1980, Law, Hollinshead et al. 2002). When

CEVs are released from the cell they are known as extracellular enveloped virus (EEV). They mediate longer-range dissemination in vitro and in vivo. Therefore CEV and EEV forms are crucial for virus dissemination (Roberts and Smith 2008).

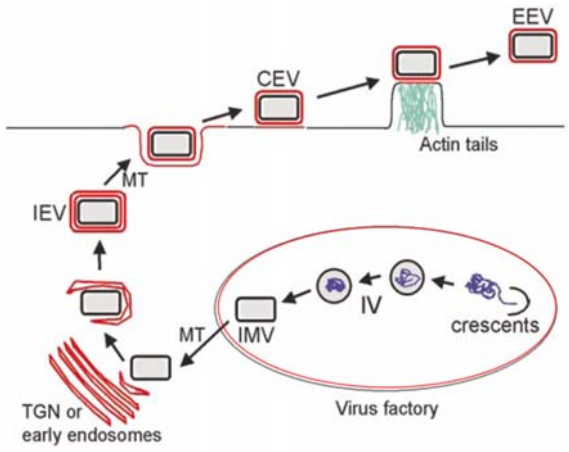


Figure 1.1 Morphogenesis of VACV. *After maturing in the virus factory, the IMV either remains in the viral factory until cell lysis or are transported via microtubules to a site near the MTOC to form IEV by double wrapping. IEV particles are transported to the surface via microtubules where the outer membrane fuses with the cell membrane to expose the CEV on the cell surface. CEVs either induce the polymerisation of actin beneath he plasma membrane or are released as EEVs.*

The IMV, surrounded by one membrane, enters cells via fusion of this virus membrane with the plasma membrane, or via macropinocytosis followed by fusion with the endosomal membrane. Fusion of the virus membrane with the cell membrane is catalyzed by virus proteins from enveloped viruses. A complex of 11 to 12 non-glycosylated, transmembrane viral proteins function in this unique fusion machinery. However CEV and EEV are surrounded by two membranes, not one, and therefore raise a topological difficulty to explain their entry. This question was answered by the discovery of a non-fusogenic mechanism mediated by specific molecules on the surface of the cell and virus which disrupted the EEV outer membrane and exposed the IMV particle within to the plasma membrane. The consequent process of entry then resembles the free IMV particle. In addition, the CEV/EEV outer membrane remains over the IMV particle as a shroud and protect the IMV particle from antibody. Once a virion has gained entry into a cell, the VACV lifecycle is initiated and protects, translation and DNA synthesis occur.

1.5 Virus gene transcription and translation

VACV transcription and translation occurs in the cytoplasm. The core of the infecting virion enters the cytoplasm and is transported on microtubules to a perinuclear location, known as the viral factory. The core of the poxvirion contains a complete viral transcriptional system including capping enzyme, poly A polymerase, early transcription initiation factor and early transcription termination factor (Condit, Moussatche et al. 2006). The virus genome is transcribed into early mRNAs by the virus-associated DNA-dependent RNA polymerase; these early mRNAs encode proteins such as the viral DNA polymerase, and enzymes to increase the size of the nucleoside pool.

After the onset of early protein expression the DNA genome is released for replication. The pattern of transcription changes to intermediate genes. Then late genes once the replication of virus DNA is finished. Generally, late genes encode structural proteins, additional virulence factors, or enzymes that are packaged into new virions to aid early gene transcription in the next cycle of infection. (Broyles 2003).

1.6 Host cell interaction during VACV morphogenesis.

Poxviruses undertake a complex replication cycle within the cell cytoplasm, involving many virus-host interactions. Two examples are the interaction between the virus and cellular cytoskeleton, and between the virus and NF-B pathways.

Microtubules and actin are fundamental structures of the cytoskeleton and both facilitate the transport of the poxvirion at different points in its cytoplasmic life cycle. When infectious virus enters the cell, VACV viral protein A10L and L4R play a role of MAP-like (microtubule-associated protein) properties and mediate interactions of the invading viral core with microtubule, which facilitates virus achieve a peri-nuclear localization. Later in the life cycle newly assembled IMV particles are transported from factories to near MTOC (microtubule organizing center) via microtubules. Subsequently, the IEV moves to the cell surface to form CEV again using microtubules. The CEV proteins A36R stimulates the formation of actin tail, which aids their particles cell-to-cell spreading.

Nuclear factor kappa B (NF-κB) is an important transcription factor for the expression of many pro-inflammatory molecules. NF-κB is retained in the cytosol of resting cells bound to the inhibitor of kappa B alpha (IκBα). Phosphorylation, ubiquitination and degradation of IκBα releases NF-κB which then translocates into nucleus to activate immune response genes. Upstream of IκBα there are different signaling pathways, such as the interleukin (IL)-1 receptor with IL-1β, tumor necrosis factor (TNF) receptor with TNF-α, or Toll-like receptors (TLRs) with their respective-ligands. These pathways are widely hijacked by VACV virus proteins, including A46, A52, N1, B14, M2, K1 and E3, resulting in inhibition of NF-κB immune response in vivo (Ember, Ren et al. 2012). Protein A46 contains a Toll-like-interleukin-1 resistance (TIR or IL-1) domain which targets the host TIR adaptor on TRIF, myeloid differentiation factor 88 (MyD88) and Toll-like receptors. Protein A52 targets both TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 2 (IRAK2) and inhibits NF-κB activation downstream of TLRs and the IL-1 receptor. Protein B14 binds to inhibitor of kappa B kinase (IKK)β, upstream of IκBα and thereby inhibits consequential phosphorylation of IκBα. Viral protein M2 directly inhibits NF-κB pathway by preventing p65 nuclear translocation and also interferes with MEK-directed phosphorylation of ERK2 and thereby partially inhibit VACV-induced NF-κB activity. Proteins K7 and K1 inhibit TLR-induced signalling (K7) and prevent IκBα degradation (K1), respectively. Finally, protein E3 inhibits NF-κB activity in both dsRNA-dependent protein kinase PKR-dependent and -independent manners (Myskiw et al., 2009), and also by antagonizing the RNA polymerase III–dsDNA-sensing pathway .

1.7 A pro-viral factors

Small interfering RNA (siRNA) screens are a good way to identify host proteins involved in virus interactions. A range of pro and anti-viral host factors (HF) were identified through an siRNA screen carried out in Dr Beard's laboratory, which found 300 cellular proteins which significantly influenced the replication and spread of VACV(Beard, Griffiths et al. 2014). Many of the pro-viral HFs were involved in translation of mRNA, while a number of anti-viral proteins played roles in cellular transcriptional processes and DNA repair pathways. A number of the cellular proteins detected had never before been linked with poxviral replication including MAST3 and nucleoporins (NUPs).

1.8 MAST3

The MAST or microtubule-associated serine/threonine kinase family contains four members (MAST1-4). MAST1 is most expressed in the brain. At neuromuscular synapses, MAST1 binds to TRAF6 and inhibits the ubiquitination and therefore activation of NF-κB, playing an important role in regulating inflammatory responses (Xiong, Li et al. 2004). The MAST2 protein has been found playing a role in the NF-kB pathway via the binding and stabilization of TRAF6 (Zhou, Xiong et al. 2004). MAST3 is predicted to contain a serine/threonine protein kinase domain near the N terminus which accounts for phosphorylation of the OH group of serine or threonine residues in substrate proteins. A PDZ protein binding domain located at the C terminus of the 143kDa peptide. Previous studies suggested MAST3 is a modulator of the inflammatory response by maintaining the activity of the TLR4-mediated nuclear factor kappa B (NF-kB) pathway (Labbe, Goyette et al. 2008). A later study identified a group of genes that are modulated by MAST3. These included a number of pro-inflammatory genes including pro-inflammatory cytokines, regulators of NF-kB, interferon inducing that defense against pathogen invasion and genes involved in cell adhesion and/ or migration (Labbe, Boucher et al. 2012).

1.9 Nuclear pore complex (NPC) and nucleoporins (NUPs)

Two VACV siRNA screens (Sivan and Beard) found nucleoporins (NUPs) as pro-viral host factors of VACV replication. This is interesting since VACV independent of the cell nucleus until late in the replication cycle when virions are undergoing the final stages of assembly (Pennington and Follett 1974, Hruby, Guarino et al. 1979). It is unclear which maturation process is facilitated by the nucleus or how this facilitation is achieved.

The nuclear pore complex (NPC) is the gateway between the nucleus and cytoplasm in eukaryotic cells. It is comprised of about 30 distinct proteins, termed nucleoporins (NUPs) (Rout, Aitchison et al. 2000, Cronshaw, Krutchinsky et al. 2002, Kau, Way et al. 2004). It acts as a transport channel, and has an eightfold symmetrical framework spanning the inner and outer membranes of the nuclear envelope. In this transport channel, unfolded domains containing FG repeats that found on NUPs are docking sites for transport receptors (Kau, Way et al. 2004). Ions and small molecules are allowed free passage through NPCs, but larger molecules over approximately 20-40kDa are actively transported through NPC by signal

identification (Rout, Aitchison et al. 2003, Suntharalingam and Wente 2003, Weis 2003, Kau, Way et al. 2004).

Most proteins that are actively transported into or out of the nucleus through the NPC require an interaction between a short amino acid motif and soluble transport receptors. The short amino acid motif on the target protein transporting in or out is termed a nuclear localization signal (NLS) or nuclear export signal (NES), respectively (Wente and Rout 2010). Importins and exportins are soluble transport receptors (generally named karyopherins) that bind to cargo through their cargo binding domain. Karyopherins also contain a NPC-binding domain(s) and an N-terminal-binding domain for the small GTPase Ran. Ran alternates between GDP- and GTP- bound states to regulate the association and dissociation of the transport receptor-cargo complex (Lee, Cansizoglu et al. 2006, Wente and Rout 2010).

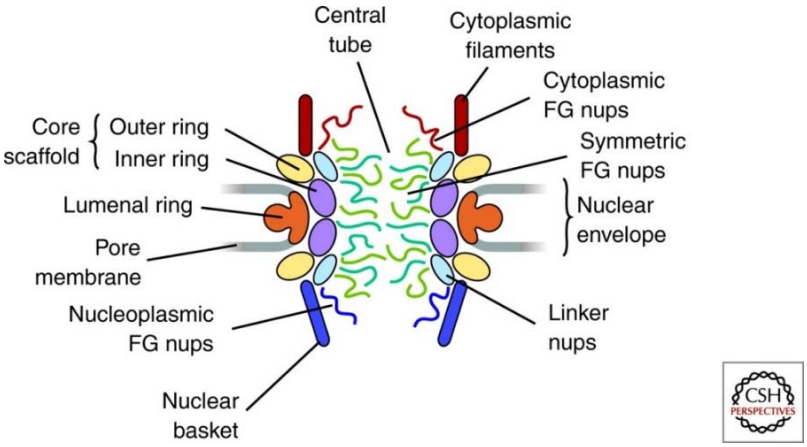


Figure 2. Major structural features of the NPC (based on the architectural map of (Alber, Dokudovskaya et al. 2007)

1.10 Nuclear export

There are two major nuclear export pathways. One depends on CRM1 (chromosome region maintenance 1, also known as exportin 1) and the other on NXF1 (also known as TIP associated protein TAP). Gene expression requires nuclear export pathways since mRNA must be exported from the nucleus. The NXF1 pathway is the main mRNA export receptor in humans (Fornerod, Ohno et al. 1997, Stade, Ford et al. 1997, Gruter, Tabernero et al. 1998). It binds with the target mRNA and recruits export receptors to form the transcription-export

(TREX) complex. This complex also consists of multisubunit THO complex, UAP56-ATP (also known as HEL) export factors and REF (also known as ALY). UAP56, an ATP-dependent RNA helicase, plays a role in mRNA splicing and TREX complex assembly. UAP56 binds REF once attained ATP and facilitates the interaction of REF and mRNA (Melchior 2001).

1. 11 Nuclear import

The import of proteins into the nucleus involves the importin-β receptor. Importin-β interacts with the amino acid sequence of NLS (nuclear localization signal) indirectly and requires the participation of other proteins which are members of importin-α family. Importin-α directly recognize NLS by NLS-binding domain that contain 10 arginine-rich motif repetitions. Although importin-α directly interact with NLS, the formation of importin-α and importin-β complex facilitates the recognition of NLS containing protein. Importin-α interacts with importin-β through the importin beta binding (IBB) domain localized in the N-terminal region of importin-α. Interaction between importin-β and the FG domains of nucleoporins on NPC initiates the translocation of import complexes. Once in the nucleus, the presence of RanGTP dissociates the import complexes, which releases the cargo. The RanGTP- importin complex exports back to the cytoplasm where importin is released by RanGAP mediated GTP hydrolyzation for another cycle (Melchior 2001).

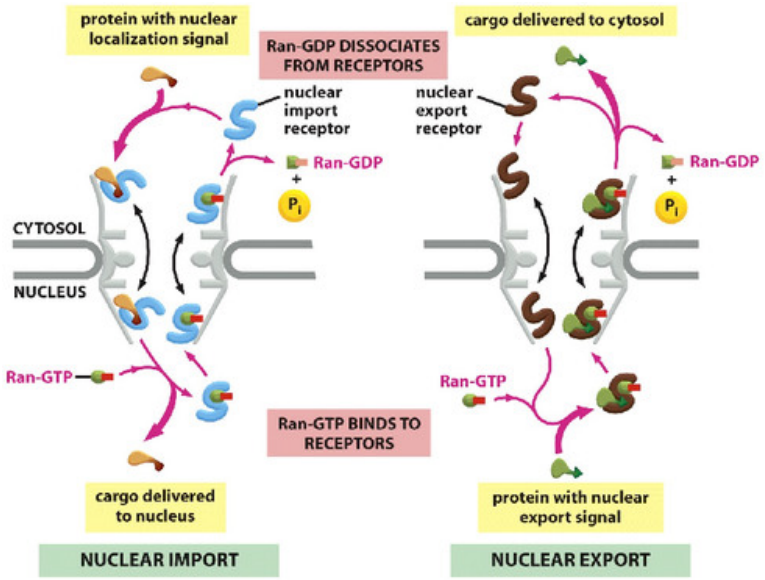


Figure 3. The RanGTPase cycle in nuclear protein import and export (Melchior 2001). *Ran is important in translocation of protein, including RanGAP1 (GTPase-activating protein1)-mediated GTP hydrolysis in the cytoplasm and RCC1 (Regulator of chromosome condensation 1) mediated conversion of RanGDP to RanGTP in nucleus. Importin β carries the NLS-containing protein into the nucleus by binding NLS motifs. The cargo is released as RanGTP binds to importin β in the nucleus. A RanGTP-importin β complex is formed. This complex then translocate into cytoplasm and release importin β by RanGAP1-mediated GTP hydrolysis, which provides free importin β for cargo in another cycle. Finally, Ran-GDP re-enters the nucleus where it is converted back to RanGTP by RCC1. Notably, exportin CAS, an importins export receptor, is essential in export of the RanGTP-importin β complex. In the cycle of nuclear export, RanGTP in the nucleus binds with the nuclear export receptor exportin. Noticeably, exportin has two binding sites therefore it can bind both cargo and RanGTP. This complex translocates onto the cytosolic side of the NPC where RanGAP stimulates the hydrolysis of GTP to produce RanGDP. Cargo and RanGDP then release from exportin. After that, exportin returns the nucleus for another cycle.*

1.12 Viral subversion of nuclear transport

During infection, viruses can disrupt nuclear transport pathways to manipulate cellular processes and suppress the host's antiviral mechanisms, thereby facilitating viral replication. Human rhinovirus (HRV) and polivovirus (PV) are cytoplasmic replicated positive strand RNA viruses. In order to inhibit host antiviral defense pathways PV and HRV can express a viral 2A protease (2Apro) to disrupt the translocation of important cellular proteins involved in immunity. 2A protease (2Apro) expressed during infection leads to the proteolytic degradation of several nucleoporins, including NUP62, NUP98 and NUP153 and possibly other factors (Gustin and Sarnow 2001, Gustin and Sarnow 2002, Park, Katikaneni et al. 2008, Castello, Izquierdo et al. 2009, Park, Skern et al. 2010, Watters and Palmenberg 2011). Mislocalization of cellular protein is also observed during PV and HRV infection of the mammal cell, which required the Kapα/Kapβ1 import pathway (Gustin and Sarnow 2001, Gustin and Sarnow 2002).

Encephalomyocarditis virus (EMCV) is another positive stranded RNA virus that interferes nucleocytoplasmic transport. By hyperphosphorylating the NUPs, EMCV leader (L) protein can alter NUP62, NUP 153 and NUP214 in an L protein-dependent manner, modifying the integrity of receptor-cargo complex (Porter and Palmenberg 2009). EMCV leader protein also suppresses the activity of RanGTPase, which strikingly inhibits the dissociation of cargos and transport factors (Porter and Palmenberg 2009).

Host invasion by pathogens elicits nucleus import of signal transducer and activator of transcription 1 (STAT1) to bind to interferon stimulated response elements (ISRE) expressed by interferon (IFN)-inducible genes (Shuai and Liu 2003). The viral open reading frame 6 (ORF6) protein is expressed by the severe acute respiratory syndrome coronavirus (SARSCoV), a positive-strand RNA virus. SARS-CoV inhibits STAT1 nuclear translocation via ORF6 protein without affecting its phosphorylation. During infection, the nuclear import factors Kapα2 and Kapβ1 are binded to the ER/Golgi membrane by which SARS-CoV ORF6 C-terminus effectively blocks STAT1 nuclear transport (Kopecky-Bromberg, Martinez-Sobrido et al. 2007, Hussain, Perlman et al. 2008).

Herpes simplex virus is a DNA virus that interferes with protein nuclear import via the Kapα/β1 and Kapβ2 nuclear import pathways. These transportins are associated to the NPC through direct interactions with NUP62 (Malik, Tabarraei et al. 2012). Although HSV-1 drastically affects host nucleocytoplasmic trafficking via viral protein ICP27, viral mRNAs can still be exported via mRNA export NXF1/TAP pathway by directly interacting with the nuclear export factor Aly/Ref (Chen, Sciabica et al. 2002).

Many DNA viruses selectively inhibit host mRNA export from the nucleus, while still retain the activity of efficient viral mRNAs export. Adenoviruses (AdVs) are double-stranded DNA animal viruses. E1B-55K and Ad E4 open reading frame 6 (E4orf6) are two adenoviral protein products that account for inhibition of host mRNA nucleus export and promotion of late viral mRNA nucleus export (Woo and Berk 2007, Blanchette, Kindsmuller et al. 2008). This occurs after E1B-55K and E4orf6 interacting with host proteins which forms a complex with E3 ubiquitin ligase activity. One study suggested this complex promotes the degradation of an unidentified cellular protein involved in host mRNA export via ubiquitin ligase activity (Woo and Berk 2007). Different opinion proposed that interaction of E1B-55K and E1B-AP5 prevents RNP E1B-AP5 interacting with NXF1, resulting in disruption of NXF1-mediated host mRNA export (Bachi, Braun et al. 2000). Evidences above showed different role of viral protein within the facilitation of viral replication and inhibition of host antiviral immune responses through mediating nucleocytoplasmic trafficking.

1.13 Poxviruses and nuclear transport

DNA viruses usually must enter the host nucleus to replicate. However a select groups of DNA viruses including poxviruses, asfarviruses and mimiviruses are the exception to this rule and replicate entirely or almost entirely in the cytoplasm. The role of the cell nucleus in poxvirus replication is unclear. Poxviruses are often described as being able to replicate "independent of the nucleus". However, careful reading of early papers on the subject show this is not true. VACV transcription, translation and DNA replication can all occur in enucleated cells, however there is a block late in virion maturation which prevents production of infectious progeny from these enucleated cells (Pennington and Follett 1974, Hruby, Guarino et al. 1979). The mechanism by which the cell nucleus enables VACV virion construction is not known, however a recent siRNA screen in the Beard laboratory found NUP98 to be a pro-viral host factor (Figure 1.4) and another VACV siRNA screen (Sivan, Martin et al. 2013) identified that the nuclear export protein nucleoporin 62 (NUP62) is also required. In this second study, knockdown of NUP62 reduced the production of infectious VACV in a one-step growth experiment, without affecting virus entry and only modestly restricting on DNA replication and early and late viral gene expression. The application of the chemical leptomycin B, an inhibitor of nuclear export, to VACV infected cells was also found to prevent virus replication. This evidence suggests nucleocytoplasmic transport, and particularly CRM1-dependent nuclear export pathways, are involved.

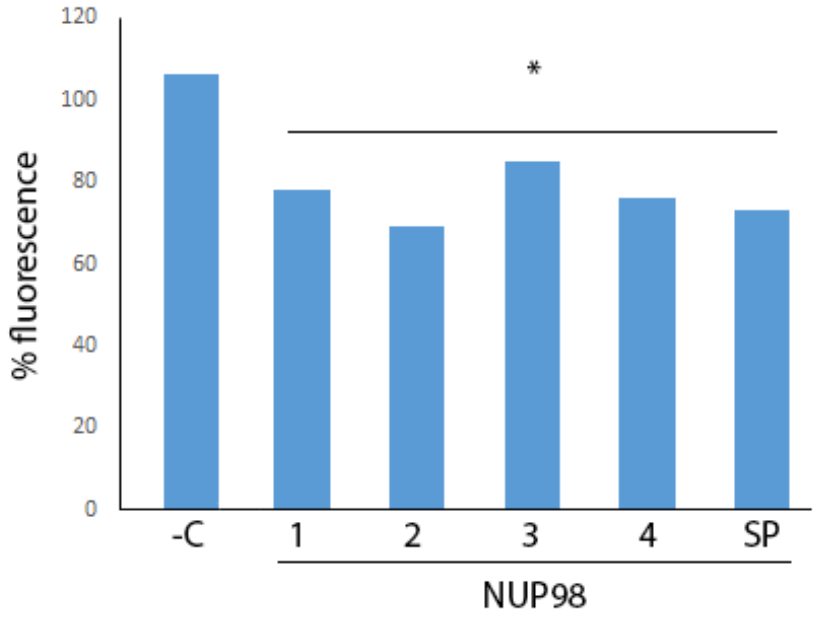


Figure 1.4 siRNA targeting NUP98 significantly reduces the level of VACV-A5-EGFP expressed fluorescence. *The NUP98 siRNA SMARTpool (SP) was separated into the four component siRNAs, each targeting different regions of the gene, and tested for their effect on VACV replication. HeLa cells were transfected with the siRNA and after 48 h the cells infected with a EGFP-tagged VACV strain and the level of fluorescence produced compared to that present in cells transfected with non-specific siRNA (-C). The percentage difference in fluorescence compared to the non-specific siRNA is shown on the y axis. Statistically significant differences are labelled (*). Data is adapted from (Beard, Griffiths et al. 2014)*

1.14 Aims of this project

This study aims to:

1. Determine the mechanism of function of MAST3 in VACV replication
2. Elucidate the role of nuclear export on VACV maturation

2. Methods

2.1 Cell and viruses.

HeLa cells, adenocarcinoma human alveolar basal epithelia cells (A549), Human Embryonic Kidney 293 cells (293T), Rabbit Kidney (RK-13), African green monkey kidney epithelia cells (Vero), African green monkey kidney cell line (BS-C-1) and MDCK cells (a kidney cell line derived from adult female cocker spaniel) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 50IU/ml penicillin, 50µg/ml streptomycin (Sigma) and 10% fatal calf serum (FBS). Cells were incubated at 37°C in a 5% CO_2 humidity incubator. Recombinant VACV tagged with enhanced green fluorescent protein (EGFP) to the VACV core protein A5 (Carter, Rodger et al. 2003) and Western Reserve (WR) strains were kindly provided by Geoffrey L. Smith (University of Cambridge, Cambridge, United Kingdom). Influenza virus PR8 were provided by Paul Digard.

2.2 Growing and purifying viral stocks.

To grow enough amount of virus, 5 T175 flasks of RK-13 were seeded in DMEM containing 10% FBS and antibiotics to get 90% confluency for each virus strain. WR and VACV-GFP viruses stock were diluted in DMEM containing 2.5% FBS and antibiotics, and infected Cells separately at 0.1 MOI with and incubated 48 hours before collected into 50ml Eppendorfs. Cell collection were centrifuged at 3000g for 10 min. The cell pellets then were re-suspended in 50ml PBS and spun down again at 3000g for 10 mins. Cells were re-suspended in 10 mM Tris-HCl pH9 and incubated on ice at least 15 min. Cells were homogenized with Dounce homogenizer and transferred to a 13.5ml screw capped centrifuge tube and spinned at 2000g for 5min. The supernatant was removed into a fresh falcon tube on ice by aspirating and trickled down the side of the tube on top of the sucrose (36 % w/v) to form a nice layer. The tubes were filled and balanced with 10 mM Tris-HCl and spinned in SW28 rotor ultracentrifuge at 13500rpm, 80min. The pellet then re-suspended in 1ml Tris-HCl pH9 and split into 1.5 screw capped tube with 350 µl for each.

2.3 SiRNA transfection.

All siRNAs were stored in 1 x siRNA buffer (Dharmacon/Thermo) at -80□C at a concentration of 20µM, and diluted to 250nM to make a working stock. In order to transfect the siRNA into cells, HeLa, Vero, A549 and 293T were seeded on 96-well plate at 5x104

cells/ml, 5x104 cells/ml, 1x105 cells/ml and 5x104 cells/ml respectively, and incubated overnight in DMEM containing 10% FBS to produce a 70%-80% confluent monolayer. Media was then removed and 80µl DMEM containing 5% FBS without antibiotics added to each well. The sequences of the DC siRNAs were as follows:

Table 1. Sequence of siRNA targeting MAST3, PRK-AB1 and VP16.

siRNA	sequence
MAST3 DC1	GAGACCAUCAAACUCAUUA
MAST3 DC2	GCGCCACGCUCCUGAAGAA
MAST3 DC3	GAAGAAUCGUCCACAGAGA
MAST3 DC4	GGACAAGUCAUCUGUGUUU
PRK-AB1	CAGAAGCCACAAUAACUUU
VP16	GGGCGAAGUUGGACUCGUAUU

10 µl Dharmafect 1 transfection reagent (Dharmacon/Thermo Scientific) was diluted in 650 µl DMEM without serum and antibiotics to make a final concentration of 1.5% and incubated for 5 minutes. For each well, 10µl of the diluted Dharmafect 1 transfection reagent was added to equal amount of siRNA and incubated for 20 min. Finally 20 µl of the siRNA / Dharmafect 1 mixture was added to the cells, making a final concentration of siRNA of 25nM, and incubated for 48 h at 37°C.

Transfection also conducted on 6-well plates. 1 x105 cells in 2ml HeLa cells were seeded per well and incubated for 24 h at 37°C. Before transfection, media was removed and replaced with 1600 µl per well DMEM containing 5% FBS without antibiotics. 10 µl diluted (5 µM solution of) MAST3 and DUSP3 small interfering RNA (siRNA) SMARTpool and four deconvoluted (DC) MAST3 siRNAs (Sigma-Aldrich) were diluted in 190 µl of 1x siRNA buffer (Dharmacon/Thermo) separately. 2µl Dharmafect transfection reagent (Dharmacon/Thermo Scientific) was diluted in 198µl DMEM without serum and antibiotics to make a final concentration of 1%. For each well, 200µl of diluted Dharmafect transfection reagent was added to equal amount of siRNAs dilution and incubated for 20 min. 400 µl of this mixture was added to the cells to make a final concentration of 25µM siRNA, incubating for 48 h at 37°C.

2.4 Virus infection

In order to infect cells the overlying media was removed and replaced with virus diluted in DMEM containing 2.5% FBS (2.5% DMEM) and antibiotics at the indicated MOI. The virus was left on the cells for 1 h at 37°C before being replaced with fresh DMEM containing 2.5% FBS (2.5% DMEM).

2.5 Growth curves

2.5.1 Fluorescent readings

The level of fluorescence produced by the VACV-A5L-GFP virus strain was used as a measurement of virus replication. Fluorescence were measured with Synergy HT MultiMode microplate reader (BioTek).

2.5.2 plaque assay

6 well plates of confluent BS-C-1 cells were prepared. Samples to be titrated were frozen and thawed three time to release the virus from cells then a 10 fold dilution series of each sample prepared in DMEM supplemented with 2.5% FBS. BS-C-1 were then infected with the dilutions for 1 h before the inoculum was replaced with DMEM containing 2.5% FBS, antibiotics and 2% carboxymethyl cellulose (CMC) (Sigma-Aldrich) and incubated for 48 hours at 37°C. After this time the CMC-DMEM was replaced with crystal violet solution (15% methanol, 5% crystal violet (Sigma-Aldrich) in water) for an hour in room temperature. The plates were then washed with tap water and the number of plaques present counted.

Plaque assay for influenza virus, seed MDCK cells in 6-well plate at 0.5x106 cells per well (2ml per well). MDCK were infected with sample supernatant at gradient dilution and incubated at 37°C for 1 hour. The virus was removed and overlay with 2ml/well Avicell (FMC) for 48 hours at 37°C. After that, Avicell was removed and 2ml/well of 10% neutral buffered formalin (NBF) (Leica) was added in each well. After 20 mins, NBF was replaced with 0.1% toluidine blue /PBS for at least 20 min.

2.6 Protein harvesting and electrophoresis and immunoblotting.

Cells were collected and lysed in ice-cold lysis buffer (137mM NaCl, 1mM EDTA, 20MmTris-HCl pH7.4, 0.5% Triton X-100) contained protease inhibitors (150 µl/well for 6-well plate) using a scraper and incubate on ice for 30 min. Lysates were centrifuged at 12,000g for 5 min at 4°C and transfer the supernatant to a new eppendof. Cell lysates were separated by electrophoresis on 10%/8% SDS-polyacrylamide gels before transferred onto

nitrocellulose membranes. Immunoreactive bands were visualized using an Odyssey infrared system. MAST3 was detected using direct infrared fluorescence (Li-Cor). Samples were prepared by adding 5 µl loading buffer (sample buffer adding 10% Mercaptoethanol) in 15µl lysate and boiling for 5 min at 98°C. MAST3 protein was resolved on an 8% Tris-glycine SDS-PAGE and transferred to a nitrocellulose membrane. Blots were treated with Odyssey blocking buffer (Li-Cor) for 1 h before incubation with MAST3 (1:500, St John's Laboratory), DUSP3 (1:5000, ABGENT), β-actin (1:2000, AbCam) antibodies. Goat anti-mouse IgG (H+L) DyLight 680 conjugate (1 in 5000, Cell Signaling) and Goat anti-rabbit IgG (H+L) Dylight 800 conjugate (1 in 5000, Cell Signaling) were used as secondary antibodies and incubated the next day. A washing step with 10% tween-PBS was followed once each antibody incubation step was finished. Blotting membranes were scanned in an Odyssey scanner, and density of bands was quantified using Odyssey scanning software.

2.7 Compounds

Nucleozin (N270) and Leptomycin B were obtained from Sigma-Aldrich Cooperation. Novel compounds 335 was provided by Karyopharm Therapeutics Inc. Carbenoxolone was provided by Sigma-Aldrich, and used as a positive control. DMSO, solvent of Nucleozin, was obtained from Sigma-Aldrichand 335 and applied as a negative control.

2.8 Cell toxicity assay

HeLa and A549 cells were seeded on two parallel 96-well plate at 5x105 cells/ml, and incubated overnight at 37°C. Beside infection, cells were pre-treated with compounds for 1 hour in working dilution. One of the plates infected with specific MOI of virus for 1 hour and replaced with new compounds. After 48 hours, added 100µl CellTiter-Blue (Promega) for each well of 96-well plate and incubate for 1 hour. Fluorescence was read at 560Ex/590Em with Synergy HT MultiMode microplate reader (BioTek). Cell death were represented by the percentage of average of living cells in treated groups compared to the average of living cells in DMSO control.

2.9 Statistics

Student's t-Test (two tail distribution with two-sample equal variance) was used to compare data using the Excel statistical package.

3. Cellular protein MAST3 facilitates VACV replication

3.1 MAST3 siRNA causes a reduction in VACV replication in both a multi-step and single-step growth curve.

MAST3 was identified as a pro-viral protein for VACV in a siRNA screen (Beard, Griffiths et al. 2014). The aim of this chapter is to investigate in more detail the role of MAST3 in VACV replication. In initial experiments the impact of MAST3 on VACV replication was assessed using a VACV strain which expresses enhanced green fluorescent protein (EGFP) tagged to the A5 viral capsid protein (VACV-A5-EGFP). The levels of the MAST3 protein in HeLa cells were knocked down using a SMARTpool of 4 siRNAs designed to target the MAST3 mRNA transcript. After 48h the cells were infected with VACV-A5-EGFP at a low multiplicity of infection (MOI=0.1) and the levels of fluorescence was measured over the following 48h as a marker of virus replication. Two negative controls were included in the experiment: mock-transfected cells and cells transfected with siRNA targeting VP16, VP16 is a protein from encoded by herpes simplex virus type 1 and thus acts as a negative control siRNA. A positive-control siRNA targeting PRK-AB1 which is known to inhibit VACV replication (Moser, Jones et al. 2010) was also included. Cells were examined microscopically for evidence of toxic effects of the siRNA and no difference was detected between treatment groups and controls. As shown in Figure 3.1, knockdown of MAST3 using a SMARTpool of four siRNAs significantly reduced the levels of fluorescence by 23% when compared to mock transfected cells. In comparison the non-targeting control VP16 produced no significant effect on fluorescence levels, while the PRK-AB1 control significantly reduced fluorescence levels, as expected. To determine whether the reduction in fluorescence caused by the MAST3 SMARTpool was due to off-target effects, in the same experiment the SMARTpool was deconvoluted into the four constituent components. Figure 3.1 shows a significant reduction in fluorescence in response to all four individual siRNAs. This result suggests that knockdown of MAST3 causes a reduction in VACV replication.

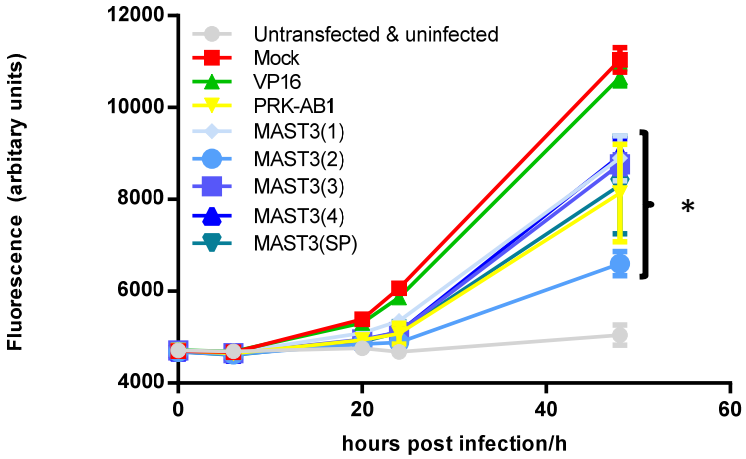


Figure 3.1. MAST3 is a pro-viral cellular protein for VACV replication in a multistep in HeLa cells.
HeLa cells were transfected with four siRNAs targeted against MAST3 (MAST3 (1-4)) and a combined MAST3 SMARTpool (MAST3 SP). In addition four controls were included: mock transfected cells, cells transfected with siRNA targeting VP16 (non-targeting siRNA), siRNA targeting PRK-AB1 (a known pro-viral host factor for VACV) and non-transfected non-infected cells. After 48h the cells were infected with VACV-A5-EGFP at 0.1 MOI. Fluorescence was measured at the indicated times using a Synergy plate reader (Biotek). Average fluorescence was calculated from 6 technical replicates and indicated for each data point. The data presented are representative of two replicate experiments. The error bars indicate standard deviation. The results were analyzed using a Student's t-Test. P values of <0.05 (compared to VP16 siRNA) are noted (*).

In order to determine the stage of the VACV life cycle that is influenced by MAST3 a single step growth curve was carried out. The experimental design was similar to that described above in the multistep growth curve, however the MOI was 5, ensuring that all cells were infected at t=0. Figure 3.2 shows that the SMARTpool siRNA and all four deconvoluted siRNAs targeting MAST3 caused a significant reduction in fluorescence. This result suggests that MAST3 is required for an early stage in VACV replication such as virus entry, transcription or translation.

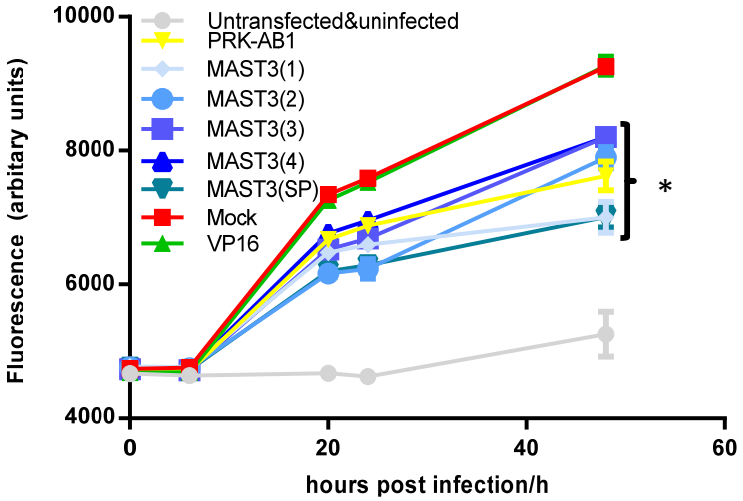


Figure 3.2 MAST3 is a pro-viral cellular protein for VACV replication in a single-step in HeLa cells. *The same experimental protocol as described for **Figure 3.1** was used, however the transfected cells were infected with VACV-A5-EGFP at a higher MOI (5). The data are representative of two biological replicates. P values of <0.05 (compared to VP16 siRNA) are noted (*).*

3.2 MAST3 antibody does not detect MAST3 on western blot

The results described in section 3.1.1 are consistent with the previous results from the siRNA screen, and suggest that MAST3 is a proviral host factor for VACV. However, it was essential to validate the efficacy of the MAST3 siRNA used in these experiments. This was investigated by immunoblotting for the MAST3 protein in HeLa cells transfected with the MAST3 siRNA SMARTpool. Briefly, cells were transfected for 48h before being collected into protein lysate buffer containing protease inhibitors, separated on a polyacrylamide gel and transferred to nitrocellulose membrane and blotted using antibody directed against MAST3. Antibody directed against actin was included as a loading control. The MAST3 gene encodes a protein of predicted size 143kDa. In Figure 3.3 actin band is visible at 42kDa and of consistent intensity showing equal loading of samples. The band was also of consistent intensity across the treatment groups with no evidence of knockdown of the protein in the MAST3 siRNA treated sample. No protein band at 143kDa was detected.

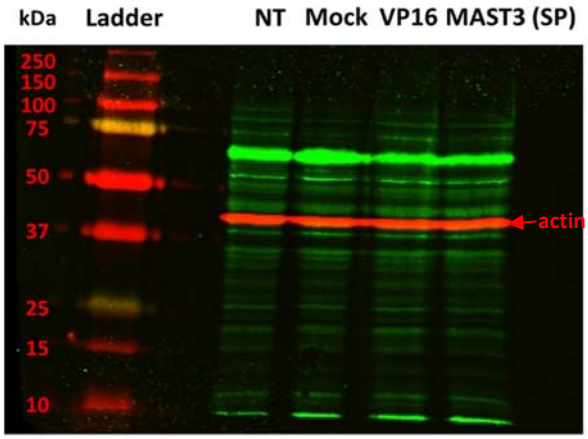


Figure 3.3. MAST3 knockdown of MAST3 protein by siRNA could not be detected by western blot.
HeLa cells were transfected with siRNAs targeted against MAST3 (MAST3 (SP)). In addition three controls were included: mock transfected cells, cells transfected with siRNA targeting VP16 (non-targeting siRNA) and cells of non-transfected. After 48h transfection, cells were collected and the level of MAST3 protein in the cell lysates was probed using western blotting with anti-MAST3 antibody and anti-actin antibody.

To determine if the 70kDa band detected by the anti-MAST3 antibody was the product of degradation the experiment was repeated using fresh protease inhibitors in the lysate buffer and keeping samples on ice between collection and loading on the gel. In addition, in order to separate the higher molecular weight protein bands more clearly the percentage of acrylamide was lowered 10% to 8% and the gel run for longer. No protein of 143 kDa was detected by the anti-MAST3 antibody however a band of 70kDa was again present, with the intensity of the band reduced in the mock lane (Figure 3.4). No protein of 143 kDa was detected by the anti-MAST3 antibody.

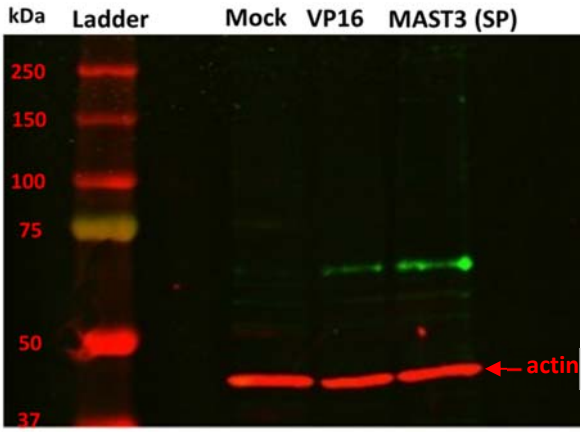


Figure 3.4. A band representing MAST3 protein of right size in HeLa cells could not be detected by western blot. *HeLa cells were transfected with siRNAs targeted against MAST3 (MAST3 smartpool (SP)). In addition controls were included: mock transfected cells, cells transfected with siRNA targeting VP16 (non-targeting siRNA). After 48h transfection, the cells were collected and level of MAST3 protein in the cell lysates was detected using western blotting with MAST3 antibody and actin antibody.*

A possible explanation for the lack of a MAST3 band at 143kDa is a mutation in the MAST3 open reading frame in HeLa cells. HeLa cells are known to be widely mutated (Landry, Pyl et al. 2013). In order to investigate this possibility the presence of MAST3 in other cell lines was investigated. Lysates of A549, Vero, 293T and HeLa cells from one well of a confluent 6-well plate were analyzed using the same antibody directed against MAST3 (Figure 3.5). Each cell line displayed same pattern of protein analysis as HeLa cells, with a strong band at 70kDa and no band at 143kDa. A large protein (approximately 250kDa) was detected in only 293T cells. This indicates that the lack of a 143kDa protein band is not a HeLa specific phenomenon.

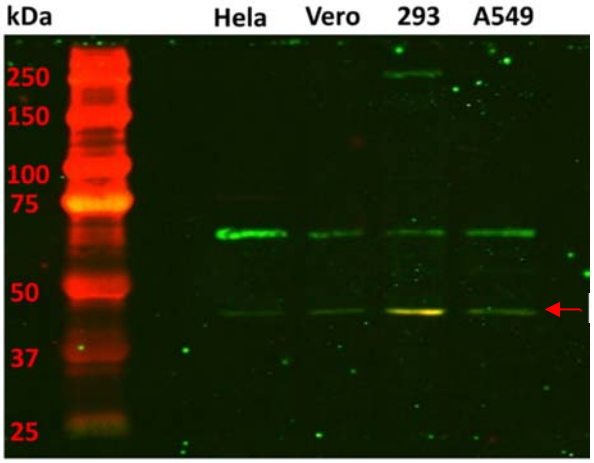


Figure 3.5. MAST3 protein of right size could not be detected, in A549, 293T, Vero or HeLa cells.
The protein lysates were collected from untransfected Hela, Vero, 293T and A549 cells. Level of MAST3 protein in the cell lysates was compared using western blotting (with MAST3 antibody; and actin antibody).

Another possible explanation for the 70kDa band detected by the MAST3 antibody is the presence of an alternative start site within the MAST3 ORF which produces a smaller size protein. The MAST3 transcripts were investigated using Ensembl (www.ensembl.org) which indicates that the full length mRNA transcript is the only protein encoding transcript produced by the MAST3 ORF (Figure 3.6) with no evidence of a smaller transcript to explain the 70kDa protein.

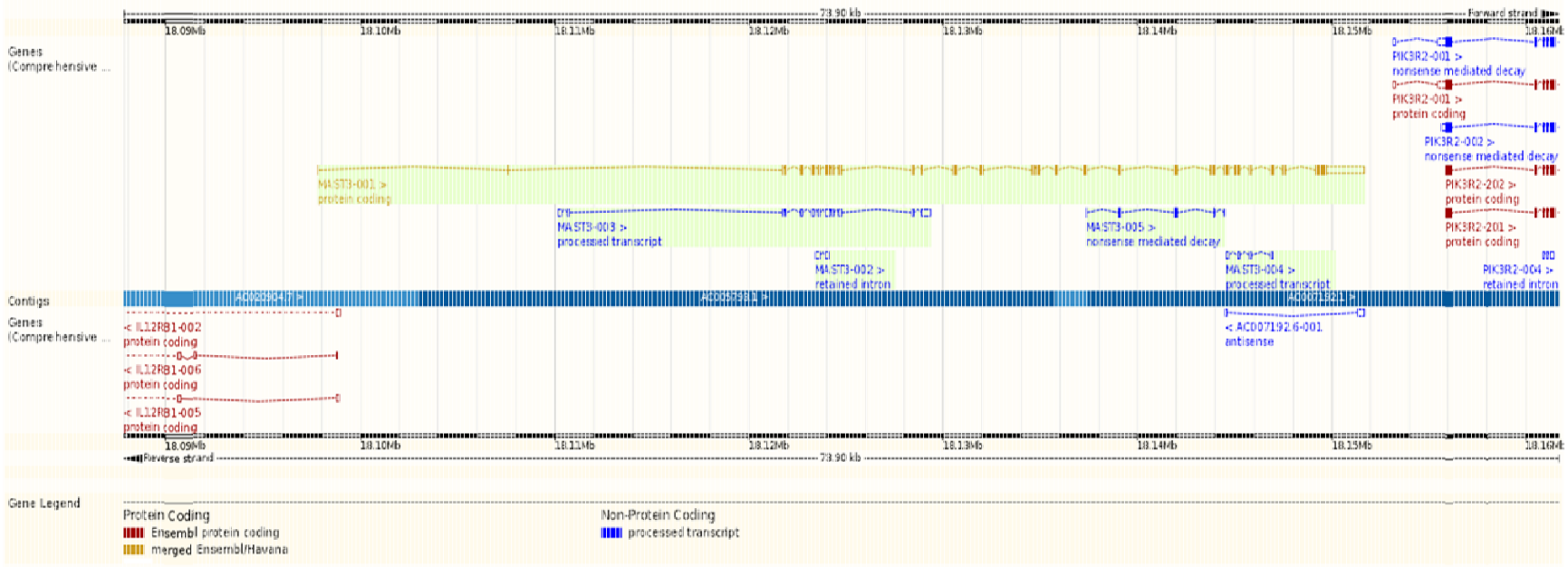


Figure 3.6 There is no evidence of an alternative, smaller MAST3 transcript. *The MAST3 ORF was examined using Ensembl (www. ensembl.org). Protein coding transcriptions are indicated in gold.*

These results suggest the 70kDa band detected by the anti-MAST3 antibody is a non-specific cross-reacting background protein. The failure of the antisera to detect a 143kDa protein on Western blot could be due to poor quality antibody. We therefore changed to an alternative method of measuring MAST3 mRNA levels is using quantitative reverse transcription PCR. In order to generate sufficient mRNA for these experiments the siRNA transfection protocol was scaled up from a 96 well plate to a 6 well plate format.

3.3 Effect of MAST3 siRNA on HeLa cells in a 6 well plate

Initially the effect of MAST3 siRNA on VACV replication in a 6 well plate was examined. Briefly, HeLa cells were plated in a 6 well plate at a confluency of 70% and transfected with siRNA targeting MAST3 (SMARTpool and deconvoluted), PRK-AB1, VP16 (at a final concentration of 25nM) or mock transfected. After 48 h the cells were either infected with VACV-A5-EGFP at an MOI of 5 or collected and stored at -70□C. The fluorescence levels in the virally-infected wells were measured over the following 48 h. This experiment was repeated 6 times and produced inconsistent results (data not shown), indicating that procedure required optimisation before this technique could be used to examine the impact of MAST3 on VACV replication. Previous work has suggested that siRNA transfection efficiency of HeLa cells drops after approximately 15 passages (Scientific 2015), therefore a new vial of low passage HeLa cells were grown up and used however the results were still inconsistent (data not shown). Repeated freeze/thaw cycles can cause degradation of siRNA therefore a new aliquot of MAST3 siRNA was used but also continued to produce inconsistent results (data not shown).

The next optimisation step was to examine the effect of increasing MAST3 siRNA concentrations. HeLa cells in a 6 well plate were transfected with a gradient of siRNA concentration from 25nM, 50nM to 100nM before infection with VACV-A5-EGFP and fluorescence measurement (Figure 3.7).

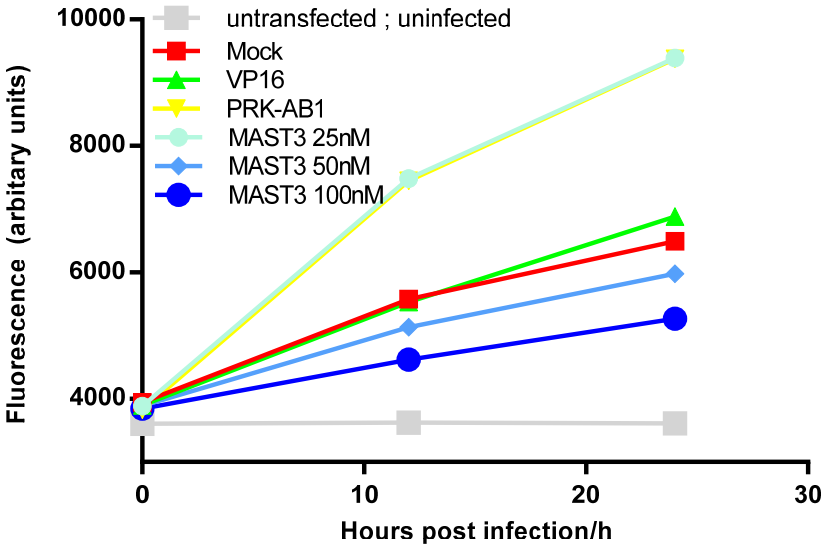


Figure 3.7. siRNA transfections in a 6 well plate format produce abnormal results.

HeLa cells were transfected with varying concentrations of siRNA targeted against MAST3 (siRNA SP). In addition, four controls were included, i.e. mock transfected cells, cells transfected with siRNA targeting VP16 (non-targeting siRNA), siRNA targeting PRK-AB1 (a known pro-viral host factor for VACV) and non-transfected& uninfected.

Increasing concentrations of MAST3 siRNA caused incremental reductions in viral fluoresence, as expected, however the fluorescence in wells transfected with PRK-AB1 siRNA (positive control) was higher than VP16 (negative control). In addition fluorescence in wells transfected with MAST3 (25nM) siRNA was higher than mock transfected cells (negative control). In the absence of the expected outcomes of the control treatments the results from this experiment could not be interpreted.

3.4 Knockdown of DUSP3 using siRNA in a 6 well plate produces variable results.

In order to optimise siRNA transfection in a 6 well plate more easily siRNA targeting the DUSP3 protein was used. DUSP3 is dual specificity protein phosphatase 3 which has previously been shown in the Beard laboratory to act as a pro-viral protein (unpublished data). In contrast to MAST3, there is a clean and strong anti-DUSP3 antibody available which allowed western blotting to be used to quantify the efficacy of siRNA knockdown. As a further trouble-shooting step a new vial of transfection reagent (Dharmafect 1) was bought

and its performance compared to the old vial. HeLa cells were transfected with siRNAs targeted against DUSP3 using new purchased Dharmafact and old Dharmafact. In addition three controls were included: mock transfected cells, cells transfected with siRNA targeting VP16 (non-targeting siRNA) and non-transfected cells. After 48h transfection, cells were collected and processed for western blot and probed with antibody against DUSP3 and actin (Figure 3.8).

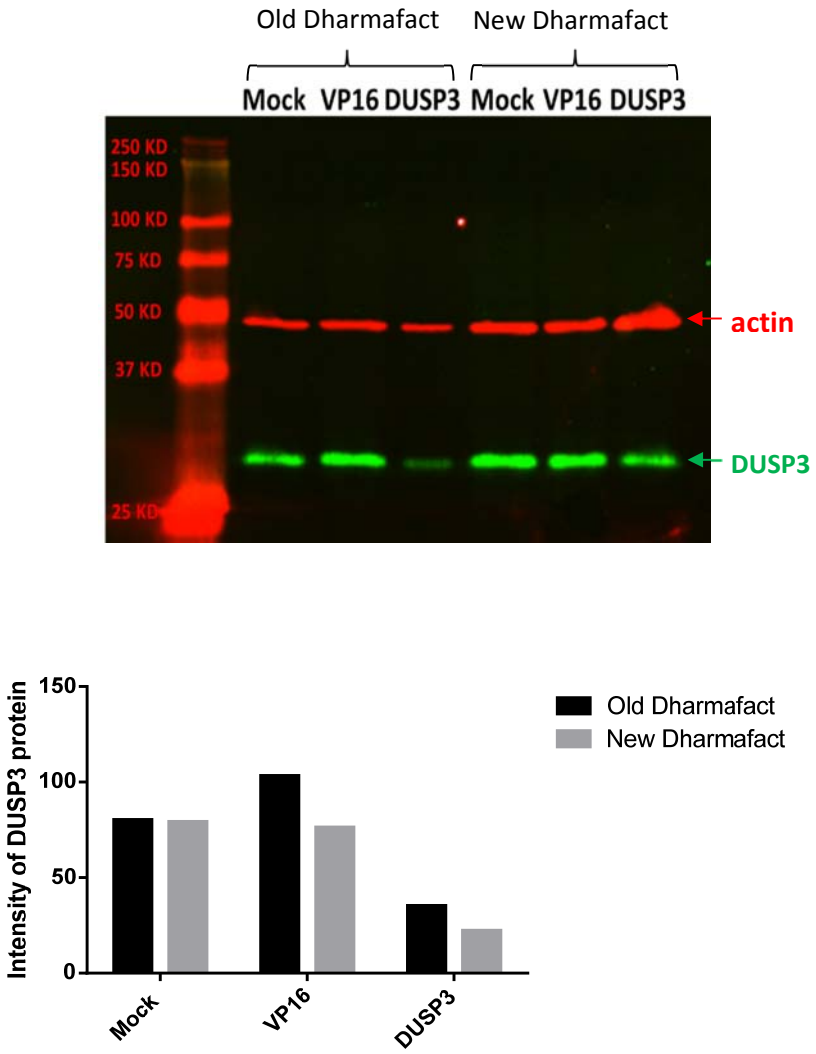


Figure 3.8. Knockdown of DUSP3 protein by siRNA in HeLa in a 6 well plate format was successfully as detected by western blot.
HeLa cells were transfected with siRNAs targeted against DUSP3 using new purchased Dharmafact and old Dharmafact. In addition three controls were included: mock transfected cells, cells transfected with siRNA targeting VP16 (non-targeting siRNA) and non-transfected cells. After 48h transfection, cells

were lysed for western blot and probed with antibody against DUSP3 and actin (A). Intensity of the bands was determined using Odyssey (Licor) software (B).

The western blot (Figure 3.8A) revealed an actin band at 49kDa in all lanes, but with some variation in intensity between samples. Licor quantification was therefore used to correct for this. Figure 3.8B shows the levels of DUSP3 were similar in the cells transfected with VP16 or mock transfected. In contrast, in HeLa cells transfected with siRNA targeting DUSP3 the levels of DUSP3 (compared to VP16 siRNA transfected cells) were 34.37% (new Dharmafact) and 28.94% (old Dharmafact), indicating the siRNA successfully knocked down the levels of DUSP3 and the siRNA protocol had worked.

The experiment was then repeated using identical conditions and reagents to assess the reproducibility of siRNA transfection in a 6-well plate format. Two additional siRNAs were included in the repeat, targeting MAST3 (SMARTpool) and PRK-AB1, in addition to a non-transfected control (Figure 3.9). In addition, there was little difference between the old and new Dharmafect reagents, showing that this was not the source of the technical problems.

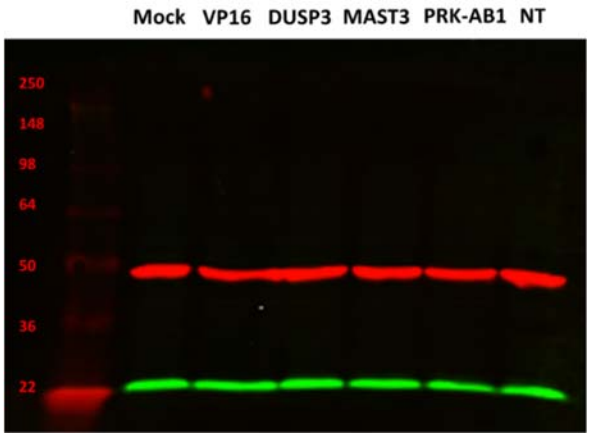


Figure 3.9. DUSP3 siRNA failed to consistently knock down DUSP3 protein levels. *HeLa cells were transfected with siRNAs targeted against MAST3 and DUSP3. In addition four controls were included: mock transfected cells, non-transfected cells, cells transfected with siRNA targeting VP16 (non-targeting siRNA) and siRNA targeting PRK-AB1 (a known pro-viral host factor for VACV). After 48h transfection, cells were lysed for western blot with antibodies targeting DUSP3 (green) and actin (red).*

In order to investigate the reproducibility of the 6 well plate siRNA transfection in more detail, two experiments were carried out at the same time in parallel by two scientists. All

experimental parameters and reagents were identical except the operator. As described previously, HeLa cells were seeded in 6 well plates, transfected with siRNA targeting VP16 (non-targeting protein), PRK-AB1 (a known pro-viral host factor for VACV), MAST3 SMARTpool, or mock transfected. After 48 h the cells were infected with VACV-A5-EGFP at an MOI of 0.1 and fluorescence measured over the following 48 h. As shown in Figure 15 the results obtained by scientist 1 (Figure 15A) were not the same as scientist 2 (Figure 15B). This confirms the inconstant nature of the siRNA transfection in a 6 well plate format.

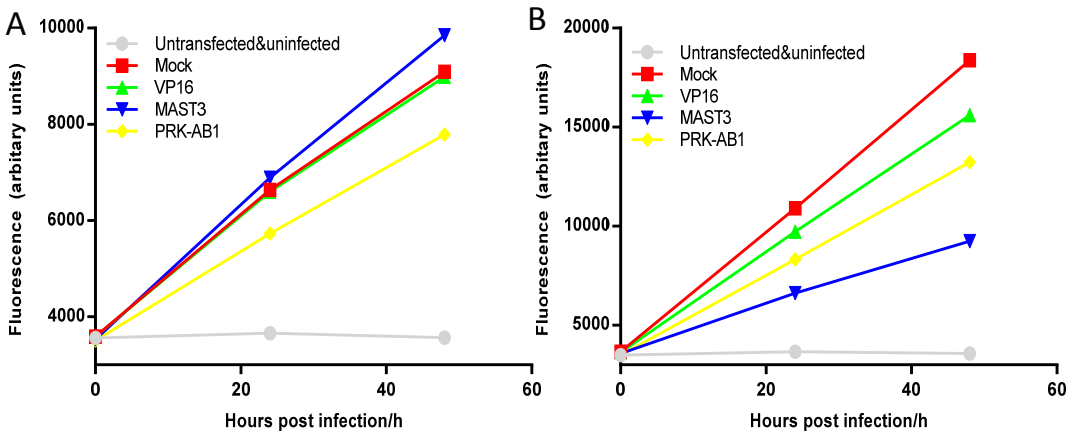


Figure 3.10 Transfection of siRNA into HeLa cells in a 6 well plate was susceptible to operator effect. *Two operators (A) scientist 1 and (B) scientist 2 conducted an identical transfection & infection experiment on 6 well plate HeLa cells. Four controls were included: cells were mock transfected, transfected with siRNA of VP16 (non-targeting protein), PRK-AB1 (a known pro-viral host factor for VACV) and cells of Non-transfected & non-infected. MAST3 SP was applied here for MAST3 protein knockdown. After 48h transfection, cells were infected with VACV-A5-EGFP at 0.1 MOI. Fluorescence was measured at the indicated time points.*

The ability to knock down protein levels using siRNA in a 6 well plate was crucial to the success of this project. Extensive troubleshooting and optimisation work had consistently produced erratic results. As a result this project was halted and research into a different topic, the influence of nuclear export on VACV replication, begun.

4. Nuclear export and VACV replication

Many DNA viruses replicate their viral genome within the nucleus, relying on nuclear proteins of the host cells for critical functions. VACV is an unusual DNA virus since it encodes a complete DNA replication, transcription and translation machinery which enables it to carry out these functions in the cytoplasm and in the absence of a nucleus (Pennington, 1974 #362, Tolonen, 2001 #364). However, the nucleus is required for assembly of the mature VACV virion. How the nucleus contributes to VACV assembly is unknown, although recent work has identified nuclear transport, in particular nuclear export, as being crucial (Sivan, Martin et al. 2013). In this chapter the impact of nuclear export on VACV replication was investigated using chemical inhibition. Four chemicals were used in the study:

- Leptomycin B is an inhibitor of CRM1, also known as exportin 1, the major nuclear export receptor. It has been shown to have significant anti-viral effect, for example against influenza virus and human immunodeficiency virus type 1 (HIV-1). LMB has shown significant toxic effects in vivo, which makes it unsuitable for therapeutic use.
- 335 is a novel compound produced by the company Karyopharm as a less toxic alternative to LMB. It has a similar mechanism of function, inhibiting CRM1-dependent nuclear export.
- Nucleozin inhibits IAV replication by two different ways. At early stages, nucleozin inhibits the synthesis of viral proteins and all classes of RNA. At later stages, once the viral ribonucleoproteins (RNPs) have formed and exited from the nucleus, nucleozin blocks the cytoplasmic trafficking of the RNPs, promoting the formation of large perinuclear aggregates of RNPs along with cellular Rab11 (Amorim, Kao et al. 2013). Nucleozin does not inhibit nuclear transport and is used as a positive control in this work for inhibition of IAV replication.
- Carbenoxolone inhibits VACV replication and was used as a positive control in this work. It has no known function on nuclear transport.

In addition the chemical DMSO, the solvent of nucleozin and 335, was used as negative "carrier" control.

The impact of the compounds in two cell lines – HeLa and A549 – was tested to ensure the changes identified were not cell-specific.

4.1 Compound toxicity.

To investigate the impact of the chemicals used in this study on cell viability, HeLa and A549 cells were treated with the panel of compounds and the level of cell viability measured. The concentrations used in the dilution series are listed in Table 4.1 and based on concentrations used in previous similar studies.

Briefly, HeLa cells and A549 cells were plated at 100% confluency and treated with varying concentrations of compounds (nucleozin, leptomycin B, 335, carbenoxolone) for 48 h. DMSO was diluted in media from 1 in 100 to 1 in 72900 in a 3-fold dilution (the same dilution protocol as nucleozin) as a carrier control. The cell viability was then determined using CellTiter-Blue (Promega) which measures the conversion of a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity, are unable to convert resazurin, and so don't generate a fluorescent signal.

Nucleozin (N270)/ µM	Leptomycin B / nM	335/ nM	DMSO v/v	Carbenoxolone/ µM
0	0	0	0	0
0.1	1	0.1	1/72900	10
0.3	3	0.3	1/24300	30
1	10	1	1/8100	100
3	30	3	1/2700	300
10	100	10	1/900	1000
30		30	1/300	
100		100	1/100	

Table 4.1. The concentration ranges used in these studies for each of the four drugs, and the dilutions of DMSO. *Nucleozin and 335 were diluted 3-fold in DMSO. Leptomycin B and carbenoxolone were diluted 3-fold in DMEM media.*

Figure 4.1 shows the percentage of viable cells in compound treated cells compared to untreated cells. In general HeLa cells were slightly more sensitive than A549 cells to the toxic effects of the compounds. In both cell lines the cell viability dropped in response to increased drug concentration, however this reached 50% cell death in only the leptomycin B treated HeLa cells at the highest concentration (100nM). For other compounds, they need higher concentration than I test to reach CC_{50}. From these results, a suitable concentration for each compound was decided, i.e. 10 µM nucleozin, 30nM 335, 30nM leptomycin B and 150 µM

carbenoxolone. These concentrations all produced less than 20% cell death on both HeLa and A549 cells.

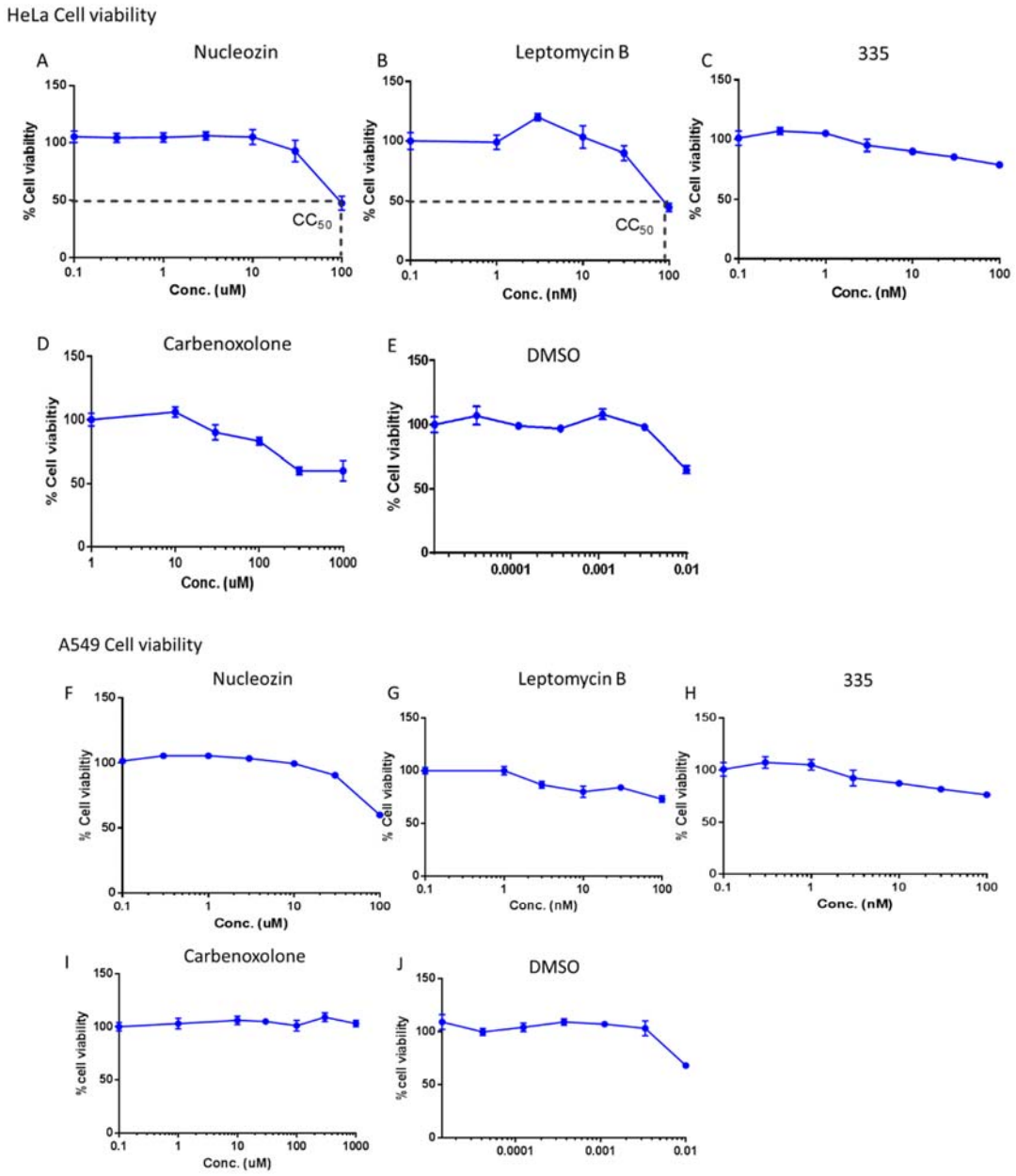


Figure 4.1 Cell viability of HeLa cell and A549 decreasing with increasing concentration of Nucleozin, Leptomycin B and 335. *HeLa cells and A549 cells were treated with serial dilution of compounds (nucleozin, leptomycin B, 335, carbenoxolone) or DMSO as a negative control and incubated at 37□C for*

48 hours. CellTiter-Blue was then used to estimate the number of viable cells present in multiwell plates via a fluorometric method. CC_{50} is indicated on the figure as a dotted line. Data shown are an average ± standard deviation of three technical replicates and representative of two biological replicates.

4.2 Nucleozin, carbenoxolone, leptomycin B and novel compound 335 inhibit IAV replication

Nucleozin, leptomycin B, 335, and carbenoxolone are all known to inhibit the replication of influenza virus A (IAV) (Watanabe, Takizawa et al. 2001, Amorim, Kao et al. 2013). To confirm that the compounds used in this study were active, their impact on IAV replication was investigated. A549 and MDCK cells were used in this experiment since IAV replicates poorly in HeLa cells. A549 and MDCK cells were pre-treated with each compound (30nM leptomycin B, 30nM 335, 10 μM nucleozin and 150 μM carbenoxolone or DMSO as a negative control) for 1 h, then infected with IAV strain PR8 at an MOI of 0.01 for 1 h (drugs were present during this time). The inoculum was then replaced with fresh media plus drug and the cells incubated for 48 h. Supernatant was then collected at 0, 24 and 48 h post infection and the amount of IAV present titred on MDCK cells. As shown in **Figure 4.2** the virus grew to high titres (over 10^7 pfu/ml) in untreated cells, as expected. The viral titre in the supernatant of cells treated with DMSO were very similar to the untreated cells, indicating that 1/100 (v/v) DMSO has no detectable impact on the replication of IAV. In contrast nucleozin treatment resulted in a clear reduction in IAV replication with a greater than 4 $\log_{10}$ reduction in virion production detected in A549 cells and greater than 3 $\log_{10}$ reduction in MDCK cells. Leptomycin B and 335 also markedly inhibited IAV replication by at least 2 $\log_{10}$ in both cell lines. Carbenoxolone also inhibited IAV replication in both A549 and MDCK cells by at least one $\log_{10}$. These results are consistent with previous studies that revealed Nucleozin, 335 and Leptomycin B all inhibited IAV replication (Watanabe, Takizawa et al. 2001, Amorim, Kao et al. 2013), and indicate that the compounds used in this study are active.

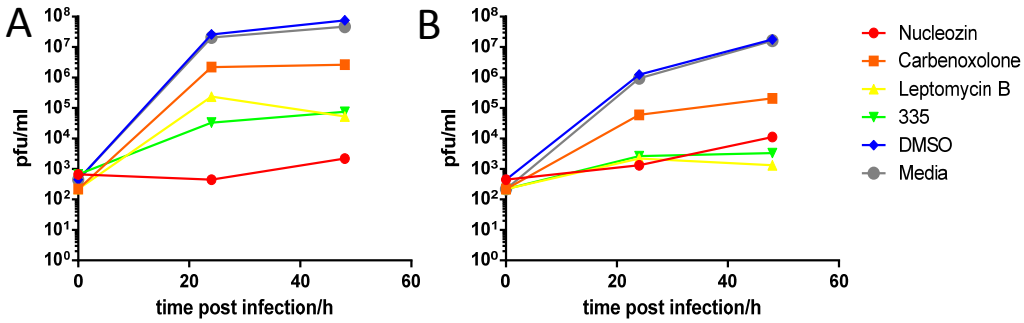


Figure 4.2 Nucleozin, carbenoxolone, leptomycin B and 335 treatment reduce influenza virus PR8 growth in A549 and MDCK. *IAV multistep growth curve in (A) A549 cells and (B) MDCK cells that treated with 10μM nucleozin, 150μM carbenoxolone, 30nM leptomycin B, 30nM 335 and 1/100 DMSO. A549 and MDCK cells were pre-treated for 1 hour with compounds followed by infection with IAV PR8 (0.01 MOI). Supernatant was collected at the indicated times and virus titred on MDCK cells.*

4.3 Impact of the compounds on VACV replication.

Having shown that the compounds exhibited antiviral activity against IAV, the effect of the compounds against VACV was investigated, with particular interest in the impact of the two nuclear export inhibitors leptomycin B and 335. Initially the impact of the compounds on the replication of the VACV-A5-EGFP virus strain was measured using the levels of EGFP fluorescence as a surrogate for virus replication. Briefly, HeLa and A549 cells were pre-treated with a range of concentrations of each compound (**Table 4.1**) for 1 h, followed by infection with VACV-A5-EGFP for 1 h during which the compounds were present. The virus was then removed and fresh media containing compounds added and the cells incubated for 48 hours.

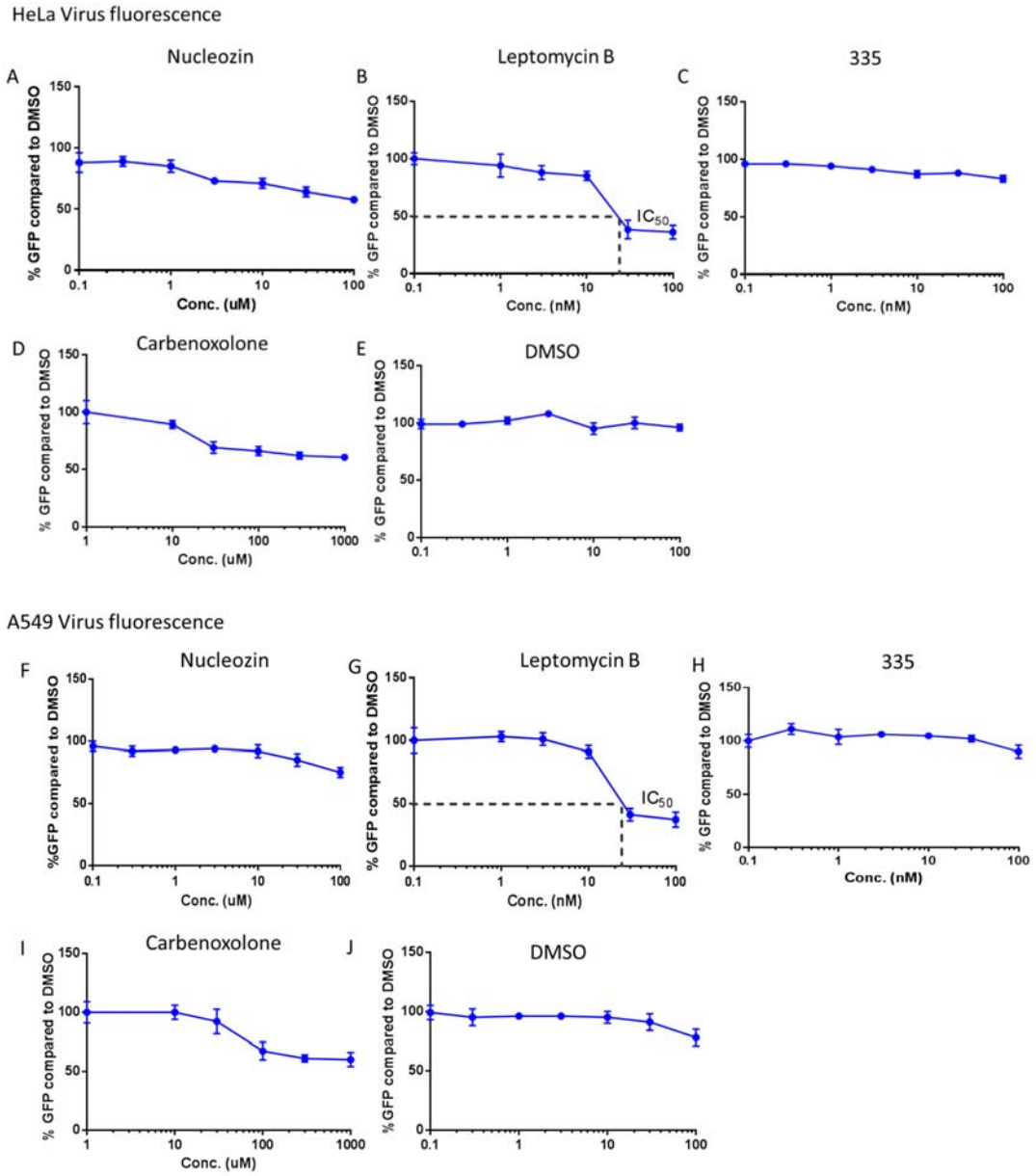
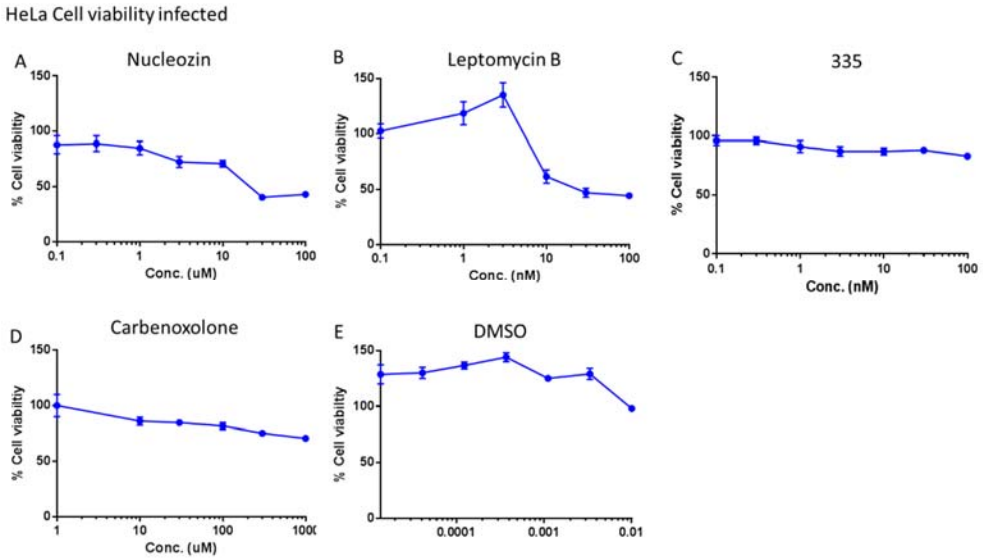


Figure 4.3 The response of VACV to a range of concentrations of nucleozin, leptomycin B, 335 and carbenoxolone in HeLa and A549 cells. *HeLa cells and A549 cells were pre-treated with compounds and followed infection with VACV-A5-EGFP and fluorescence measured.*

Figure 4.3 shows that DMSO had little effect on virus replication compared to untreated cells until high concentrations of DMSO were reached. Carbenoxolone, as expected, caused a large reduction in fluorescence levels in both cell lines at 30-100nM and nucleozin caused a gradual reduction in fluorescence as drug concentration increased. However neither carbenoxolone or

nucleozin caused a 50% reduction in fluorescence levels. As previously published, leptomycin B caused a reduction in viral fluorescence with IC_{50} of 14.69nM in HeLa cells and 15.07nM in A549 cells. Surprisingly, novel compound 335, which has the same mechanism of action as leptomycin B, had very little effect on fluorescence levels, with less than 20% reduction in fluorescence even at high concentrations. This suggested the reduction in fluorescence seen in leptomycin B treated cells may have been due to an off-target effect. Indeed, when the cells were examined under light microscopy, substantial cell death could be seen in the wells treated with higher concentrations of leptomycin B. In order to quantify the amount of cell death seen in compound-treated and virus-infected cells, CellTiter-Blue was added to the wells 48 h post infection and fluorescence read (**Figure 4.4**). The profile of cell death was remarkably similar to the profile of viral fluorescence. This was particularly striking in the case of leptomycin B.



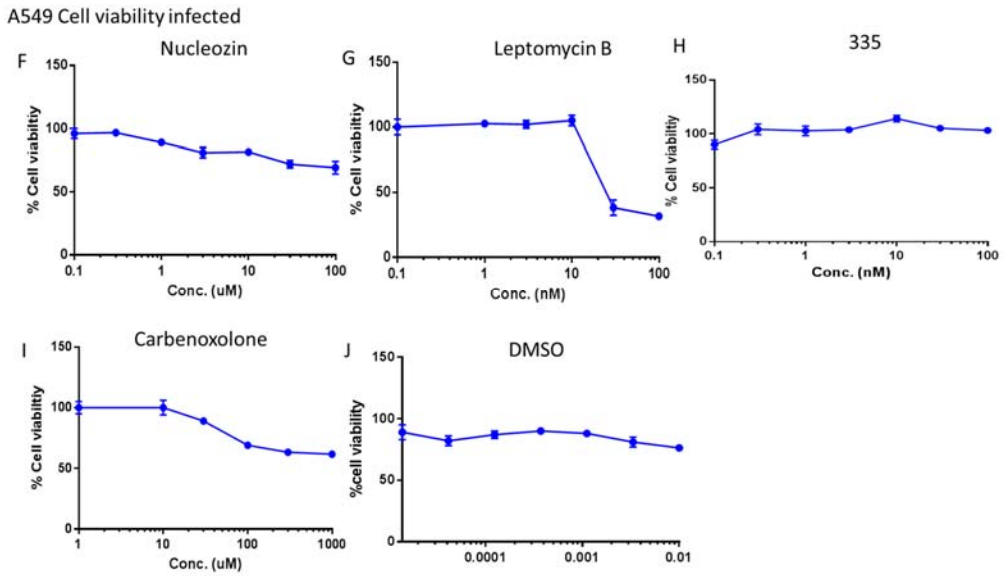


Figure 4.4 Leptomycin B causes cell death in VACV-infected cells. *HeLa cells and A549 cells were pre-treated with compounds for 1 h, followed 0.01 MOI infection with VACV-A5-EGFP for 1 h. Cells were further incubated in fresh media containing compounds for 48 h. Cell viability was measured with CellTiter-Blue. The data shown are average of three technical replicates and representative of three biological replicates. Error bar are standard error of the mean.*

Comparison of the data in **Figures 4.3** and **4.4** strongly suggest that cell death caused the reduction in virus fluorescence seen in leptomycin B treated cells, rather than inhibition of viral replication. To further investigate the accurate level of the virus reduction produced from compounds, the fluorescence levels were corrected for cell death (**Figure 4.5**).

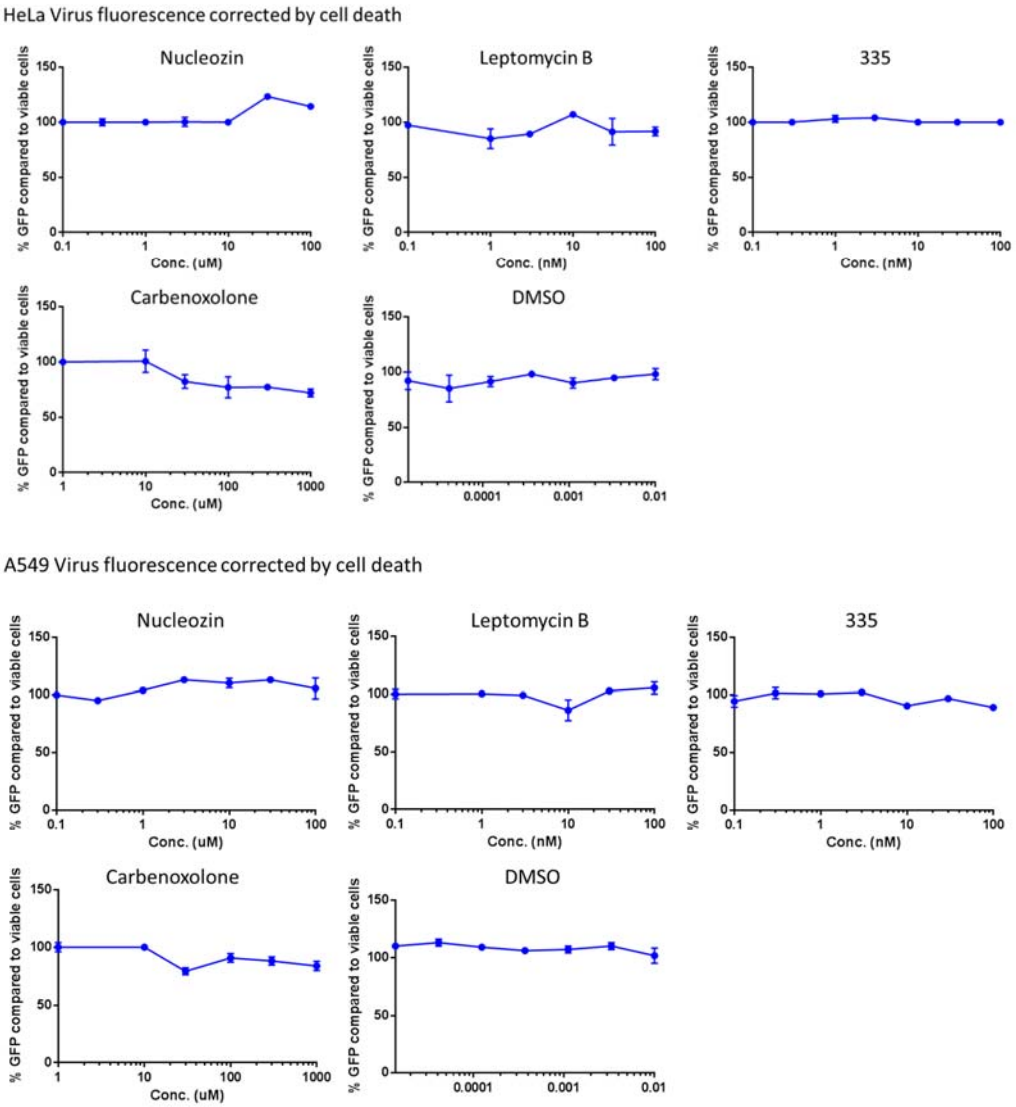


Figure 4.5 Inhibition of CRM1 nuclear export pathway does not inhibit VACV replication. *Data from **Figure 4.3** (viral fluorescence) were corrected using data from **Figure 4.4** (cell death data) to produce an estimate of the "fluorescence per cell present".*

The virus fluorescence was very similar to DMSO-treated cells for all compounds at all concentrations except carbenoxolone, indicating only carbenoxolone has an anti-VACV effect, and highlighting the importance of measuring cell death when investigating anti-viral effects. Calculating the selective index of the compounds (**Table 4.2**) is another way of determining the anti-viral effect of a compound.

Table 4.2. List of established and experimental drugs for testing in combination studies and their effect on VACV infection in vitro

Compound	Cell type	IC_{50} (µM)[1]	CC_{50} (µM)[2]	SI[3]
Nucleozin	HeLa	-	93.3	-
	A549	-	-	-
Leptomycin B	HeLa	22.69	90.3	3.98
	A549	22.69	-	-
335	HeLa	-	-	-
	A549	-	-	-
Carbenoxolone	HeLa	-	-	-
	A549	-	-	-
DMSO	HeLa	-	-	-
	A549	-	-	-

[1]IC_{50} - Represents the concentration of a drug that is required for 50% inhibition *in vitro*
[2]CC_{50} - Represent the concentration of a drug that is required for 50% cytotoxicity
[3]Selective index – CC_{50}/IC_{50}

The selective index could only be calculated for leptomycin B on HeLa cells and was 3.98. Ideally the selective index should be high, indicating the drug has an antiviral effect in the absence of any cell toxicity effects. 3.98 is a small SI and indicates leptomycin B causes antiviral and cellular toxicity at similar concentrations.

4.4 Leptomycin B has no significant effect on Vaccinia virus yield.

The previous experiments used EGFP fluorescence measurements to estimate the level of VACV replication. While this is a convenient and often-used method of determining viral growth it is not a direct measure of infectious virus production. In order to determine if leptomycin B, 335, carbenoxolone or nucleozin inhibit the production of infectious VACV virions we used viral plaque-forming assays. A549 and HeLa cells were pre-treated with compounds (30nM leptomycin B, 30nM 335, 10 µM nucleozin and 150 µM carbenoxolone) for 1 h, and infected with VACV-WR (0.01 MOI) for a multistep infection. 1 h post infection cells were further incubated in fresh media with compounds for 48 h. Cells and supernatant were collected together at 24 h and 48 h post infection and virus titrated using a plaque assay (**Figure 4.6**).

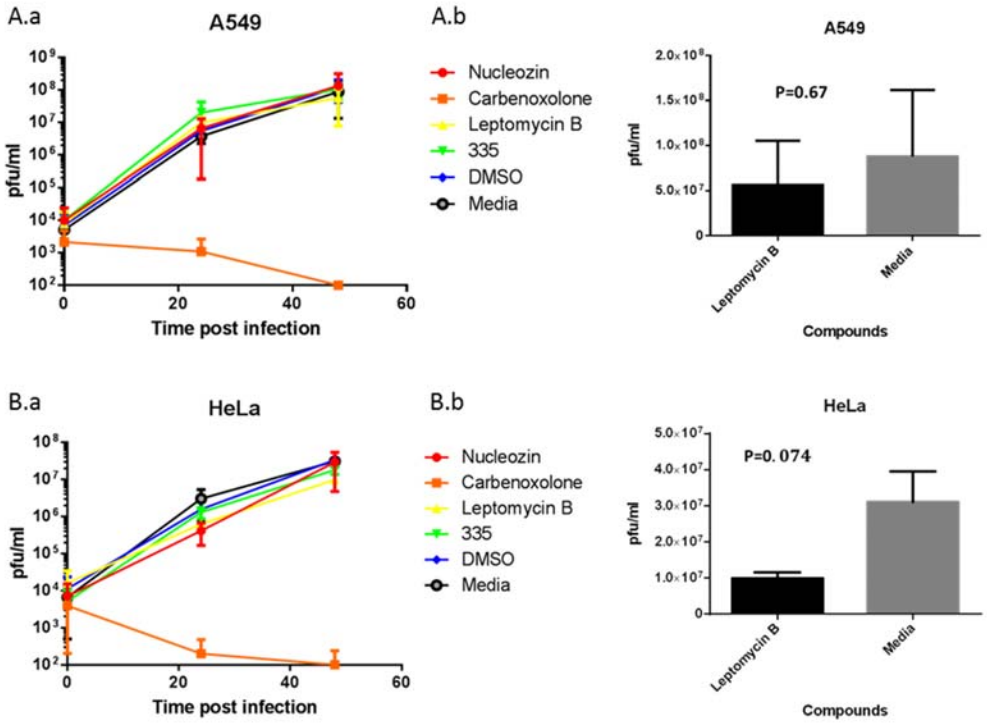


Figure 4.6 Nuclear export inhibitor have no significant effect on the production of VACV virions. *A549 and HeLa were pre-treated with compounds (30nM leptomycin B, 30nM 335, 10 μM nucleozin and 150 μM carbenoxolone, respectively) for 1 h, then infected with VACV (0.01 MOI) for a multistep growth curve. Cells and supernatant were collected together at 24-h and 48-h time point for plaque assay. Data shown were average of two biological replicates. Error bar shows the standard error of the mean.*

DMSO and untreated cells yielded high VACV titres of approximately 10^8 pfu/ml on A549 cells and over 10^7 pfu/ml on HeLa cells. VACV was completely inhibited on both A549 and HeLa cells treated with 150 μM carbenoxolone with no plaques detected at 48 h. No statistically significant difference in titre was detected in nucleozin, leptomycin B or 335 treated cells compared to DMSO or untreated controls. In particular, leptomycin B inhibited VACV replication on A549 and HeLa by 1.6 fold (p-value=0.67) and 3.3 fold (p-value=0.074) compared to untreated cells. These results confirm that neither leptomycin B or 335 have a significant effect on VACV virion production, consistent with the result from fluorometric experiments. This proves that CRM1-dependent nuclear export is not required for productive VACV replication.

5. Discussion

MAST3 was previously identified as a regulator of NF-κB activity, therefore it was a priority hit from the siRNA screen carried out in Dr Beard's laboratory. The initial hypothesis was that VACV uses MAST3 to evade host antiviral defences via inhibition of the NF-κB pathway. NF-κB is a major transcription factor which rapidly activates innate and adaptive immune responses, and is a key antiviral defence mechanism. VACV is known to evade the NF-κB pathway multiple different ways, and modulating MAST3 activity could represent another example (Labbe, Goyette et al. 2008, Labbe, Boucher et al. 2012). Initial results revealed a reduction in virally-encoded fluorescence in response to MAST3 siRNA whichwas consistent with this hypothesis. Unfortunately after these initial promising results there were technical difficulties with the project which prevented us from proving or disproving the theory.

The MAST3 antibody did not detect a protein band of the correct size (143kDa) from a range of cell types on a western blot. A lower band of 70kDa that was consistently detected by the antibody was most likely a non-specific band. The absence of a 143kDa band suggested the MAST3 protein was either expressed at levels too low to detect using this technique, or that the antibody was poor quality and unable to detect the protein. One trouble-shooting step which could be tried in the future would be to over-express a recombinant MAST3 protein from a plasmid and test whether the antibody detects the over-expressed protein. The MAST3 siRNA could then have been employed in cells over-expressing MAST3 to determine if it reduces protein expression. We chose to change to a different method of detection of protein expression and use quantitative polymerase chain reaction (qPCR) to calculate the amount of MAST3 mRNA present. This required a move to a six well plate format rather than the 96 well plate format that had been used up till then. Unfortunately the siRNA transfection method did not produce consistent results in this different format despite intensive trouble-shooting and optimisation. This project was therefore shelved due to time constraints associated with a one year research project.

If time had permitted the project to be continued, a new method of modulating MAST3 protein expression could have been used, for example CRISPR/Cas 9 genetic modification, short hairpin RNA (shRNA), or using mouse embryo fibroblasts isolated from MAST3 knockout mutant mice. Alternatively, overexpression of MAST3 on a plasmid may produce a reversed phenotype and could be used to investigate the function of MAST3 protein in VACV

replication.

The second project reported in this Masters thesis was an investigation into the role played by the host cell nucleus in poxvirus replication. Again, we used VACV as our prototype poxvirus. Poxviruses famously replicate soley in the cytoplasm (Morgan, Ellison et al. 1954), an unusual strategy for a DNA virus. Work published over 40 years ago reported that despite the cytoplasmic location of VACV replication, the virus still requiresthe cell nucleus for successful virion morphogenesis (Pennington and Follett 1974). This finding was subsequently supported by other researchers (Hruby, Guarino et al. 1979). More recently two siRNA screen papers (Sivan, Martin et al. 2013, Beard, Griffiths et al. 2014) both identified NUPs, which are involved in nucleocytoplasmic transport, to be strong proviral host factors. One paper also reported an anti-viral effect exerted by the nuclear export inhibitor leptomycin B which specifically inhibits the CRM1 export pathway (Sivan, Martin et al. 2013). This body of work suggests that VACV requires the host cell nucleus for one of the final stages of replication – the maturation of the virion particle. In order to investigate this further we used leptomycin B and another CRM1 pathway inhibitor known as 335. Initial experiments investigated the toxic potential of the compounds and then confirmed their activity by using them to reduce the replication of IAV. The effect of the compounds against VACV was then tested using a fluorescently labelled VACV strain. In these experiments the level of fluorescence was measured as a proxy of virus replication. A reduction in replication was seen in response to the positive control compound carbenoxolone and also leptomycin B, however 335 had no effect on fluorescence levels. This conflict in findings between the two CRM1 pathway inhibitors was solved when the level of cell death was examined. Leptomycin B treatment plus VACV infection caused significant cell death, whereas 335 did not. When the fluorescence levels were corrected for cell death it was clear that the original reduction in fluorescence in the leptomycin B treated, VACV infected cells was due to cell death and not a reduction in virus replication. The conclusion of this work was that the CRM1 pathway is not involved in VACV replication. This was strengthened by the final experiment which directly measured VACV replication using virus titration. The number of infectious virions from cells treated with leptomycin B or 335 and then infected with VACV (wildtype strain) was compared to untreated and DMSO treated cells and no significant different was detected.

In order to put this finding into context with the published literature the previous papers were re-examined. Pennington and Follet (Pennington and Follett 1974) carried out their work

using a very high MOI (50) which could have led to artifical results. However, Hruby and co-workers (Hruby, Guarino et al. 1979) used a MOI of 5, and described extensive control experiments to prove the enucleated cells were viable for the length of the experiment. They clearly showed that VACV can undertake transcription, translation and DNA replication in the absence of the host cell nucleus, a remarkable feat of independence. However the host cell nucleus is required much later in the VACV life cycle, to allow immature virion particles to progress to mature infectious virions. Sivan and co-workers (Sivan, Martin et al. 2013) extended this work by describing a pro-viral effect of the NUP62 protein and leptomycin B, and suggest VACV requires a functional nuclear-cytoplasmic transport and particularly the CRM1 export pathway. However when this paper was examined closely the NUP62 siRNA treatment knocked down VACV growth by a small amount, only 1/3rd of a log10. No statistical analyses were reported on the significance of this reduction. Equally importantly no cell death data was reported, therefore the potentially toxic effect of the NUP62 siRNA may not have been taken into account. The effect of leptomycin B treatment on VACV replication was reported in the same paper using an unusual method. A fluorescently labelled EGFP-VACV strain was used, and virus replication in control and leptomycin B treated cells compared by counting the number of GFP positive cells. Leptomycin B treatment reduced the number of GFP positive cells by 50%, however again no cell death data was reported and no statistical analyses included. The effect of leptomycin B on overall levels of fluorescence or the production of infectious viral particles (commonly used methods of measuring VACV replication) was not reported. In the absence of cell death measurements and statistical analyses and the unusual methods used to measure virus replication these findings must be viewed with caution.

While the NUP62 and leptomycin B data (Sivan, Martin et al. 2013) are not entirely convincing, data from the earlier enucleation studies are, and are supported by work from the Beard laboratory which showed that siRNA targeting the NUP98 protein (which is also involved in nucleocytoplasmic transport) significantly reduces VACV replication (Figure 1.4). This latter work asessed the levels of cell death caused by the siRNA transfection and corrected the fluorescence levels accordingly, and carried out robust statistical analyses on the data. Therefore there is undoubtedly evidence that VACV requires a host cell nucleus in order to complete its life cycle. The work in this thesis adds to our understanding of this subject by showing that the CRM1 export pathway is not required.

While the CRM1 transport pathway may not be required, there are alternative nucleocytoplasmic transport pathways which may be required by VACV, such as the other major nuclear export pathway (NXF1/TAP), or an import pathway. In the future, these could be examined by using siRNA to knock down crucial components of the NXF1/TAP pathway, or chemical inhibitors such as importazole which inhibits nuclear import pathways. An alternative means of reducing nucleocytoplasmic transport could be to use dominant negative Ran proteins (Kurisaki, Kurisaki et al. 2006).

The central question related to our work is why does VACV require the cell nucleus at such a late stage in its morphogenesis? Does the virus require a nuclear protein to be transported to the viral factory to assist in virion maturation? Or does the virus need to transport a viral protein into the nucleus in order to modulate a cellular pathway? It is possible that VACV inhibits nucleocytoplasmic transport at early stages in the virus life cycle in order to prevent host antiviral mRNA transcripts being trafficked into the cytoplasm and translated. Other viruses are known to employ this immune-evasion mechanism. However, later in the viral life cycle the host protein production has been very effectively shut off by the virus, and it may therefore be able to release the suppression of nucleocytoplasmic transport and use nuclear factors to facilitate the maturation of virions.

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